VALORIZAÇÃO INTEGRAL DE PLANTAS ARBUSTIVAS ESPONTÂNEAS NUM ENQUADRAMENTO DE BIORREFINARIA

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ORIENTADORA: Professora Catedrática Helena Margarida Nunes Pereira

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TESE ELABORADA PARA A OBTENÇÃO DO GRAU DE DOUTOR EM

ENGENHARIA FLORESTAL E DOS RECURSOS NATURAIS

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Aos meus filhos:

"É tão bom morrer de amor e continuar vivendo"

(Mário Quintana)

ÍNDICE

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RESUMO

As plantas arbustivas representam uma parte muito significativa do sobcoberto florestal e, consequentemente, uma fonte relevante de biomassa lenhocelulósica, mas encontram-se ainda pouco exploradas por falta de alternativas para a sua valorização industrial.

Neste trabalho, a espécie *Cistus ladanifer* (esteva), que é atualmente apenas usada para a extração de lábdano e óleos essenciais, foi utilizada como modelo para o desenvolvimento de um processo integrado de valorização da biomassa arbustiva num enquadramento de biorrefinaria.

O processo de valorização desenvolvido inicia-se com a remoção dos extrativos (40% p/p), produzindo-se uma corrente líquida rica em compostos fenólicos (maioritariamente ácido gálico e flavonoides) com propriedades antioxidantes e uma corrente sólida contendo polissacarídeos e lenhina. A corrente sólida foi submetida a processos de fracionamento seletivo para a recuperação sequencial das hemiceluloses (processo de auto-hidrólise) e lenhina (procesos organosolv e alcalino), obtendo-se finalmente uma corrente sólida enriquecida em celulose que apresenta uma digestibilidade enzimática acrescida (cerca de 4 vezes).

A otimização da auto-hidrólise permitiu obter xilo-oligossacarídeos, com potencial atividade prebiótica, numa concentração máxima de 16 g/L, a que corresponde um rendimento de 10,2 g/100 g matéria-prima. Os processos de deslenhificação estudados afetaram a composição monomérica da lenhina residual, provocando um decréscimo da razão S/G (quantificado por pirólise analítica, Py-GC/MS) e a recuperação de vários compostos fenólicos com elevado valor acrescentado, nomeadamente o ácido vanílico, ácido *p*-cumárico e epicatequina, identificados por eletroforese capilar (CZE).

Os sólidos ricos em glucana e os hidrolisados hemicelulósicos ricos em pentoses, foram utilizados em separado ou conjuntamente para produção seletiva do isómero ácido D-láctico (D-LA) pela estirpe recombinante *Escherichia coli* JU15, em diferentes modos de fermentação: hidrólise e fermentação separadas (SHF), sacarificação e fermentação simultâneas (SSF) e sacarificação e co-fermentação simultâneas (SSCF). Em todas as condições testadas, o rendimento em ácido láctico ($Y_{LA/açúcares}$) foi sempre superior 0,92 g/g.

Palavras-chave: *Cistus ladanifer*, Ácido D-láctico, Fracionamento, Oligossacarídeos hemicelulósicos, Lenhina

ABSTRACT

Natural shrubs represent a very significant part of the forest understory and, consequently, a relevant source of lignocellulosic biomass, but that are still underexplored due to the lack of alternatives for their industrial valorization.

In this work, the specie *Cistus ladanifer* (rockrose), which is currently only used for the extraction of labdanum and essential oils, was used as a model feedstock for the development of an integrated process for the valorization of shruby biomass in a biorefinery framework.

The developed approach started with the removal of extractives (40% w/w), producing an extract rich in phenolic compounds (mainly gallic acid and flavonoids) with antioxidant properties. The remaining lignocellulosic material was subjected to selective fractionation processes for the sequential recovery of hemicelluloses (autohydrolysis process) and lignin (organosolv and alkaline processes), producing a solid enriched in cellulose that has an increased enzymatic digestibility (approximatly 4 times, as compared to the feedstock).

The optimization of the autohydrolysis process allowed to obtain xylo-oligosaccharides, with potential prebiotic activity, in a maximum concentration of 16 g/L, corresponding to a yield of 10.2 g/100 g extracted feedstock. The delignification processes studied affected the monomeric composition of the residual lignin, causing a decrease of the S/G ratio (quantified by analytic pyrolysis, Py-GC/MS) and the recovery of several phenolic compounds with high added value, namely vanillic acid, *p*-coumaric acid and epicatechin, identified by capillary electrophoresis (CZE).

Glucan-rich solids and the pentoses-rich hemicellulosic hydrolysates were used, separately or together, for selective production of the D-lactic acid isomer (D-LA) by the recombinant strain *Escherichia coli* JU15 in different fermentation modes: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and simultaneous saccharification and co-fermentation (SSCF). In all conditions tested, lactic acid yield ($Y_{LA/sugars}$) was always higher than 0.92 g/g.

Keywords: Biomass fractionation, *Cistus ladanifer*, D-lactic acid, Hemicelulosic oligosaccharides, Lignin

LISTA DE PUBLICAÇÕES

Esta tese é baseada nos seguintes artigos:

I. Alves-Ferreira, J., Fernandes, M. C., Pereira, H., Carvalheiro, F. (2019). Potential use of rockrose as a feedstock for the biorefineries: a review (em submissão)

II. Alves-Ferreira, J, Miranda, I., Duarte, L. C., Roseiro, L. B., Lourenço, A., Quilhó, T., Cardoso, S., Fernandes, M. C., Carvalheiro, F., Pereira, H. (2019). *Cistus ladanifer* as a source of chemicals: structural and chemical characterization. Biomass Conversion and Biorefinery, https://doi.org/10.1007/s13399-019-00448-8

III. Alves-Ferreira, J., Duarte, L. C., Fernandes, M. C., Pereira, H., Carvalheiro, F. (2017). Hydrothermal Treatments of *Cistus ladanifer* Industrial Residues Obtained from Essential Oil Distilleries. Waste Biomass Valorization, 10, 1303-1310

IV. Alves-Ferreira, J., Duarte, L. C., Lourenço, A., Roseiro, L. B., Fernandes, M. C., Pereira, H., Carvalheiro, F. (2019). Distillery residues from *Cistus ladanifer* (rockrose) as feedstock for the production of added-value phenolic compounds and hemicellulosic oligosaccharides. Bioenergy Research, 12, 347–358

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VI. Alves-Ferreira, J., Carvalheiro, F., Duarte, L. C., Ferreira, A. R. P., Martinez, A., Pereira, H., Fernandes, M. C. (2019). D-lactic acid production from *Cistus ladanifer* residues: co-fermentation of pentoses and hexoses by *Escherichia coli* JU15 (em submissão)

A produção científica a seguir não é apresentada nesta tese, mas faz parte do conjunto do trabalho desenvolvido durante o meu doutoramento

VII. Fernandes, M. C., Alves-Ferreira, J., Duarte, L. C., Pereira, H., Carvalheiro, F., Martinez, A. (2019). D-Lactic Acid Production from Hydrothermal Pretreated, Alkali

Delignified and enzymatically Saccharified rockrose with the metabolic engineered *Escherichia coli* strain JU15 (em submissão)

Comunicações em Congressos

Orais:

I. Alves-Ferreira, J., Fernandes, M. C., Duarte, L. C, Martínez, A., Pereira, H., Carvalheiro, F. (2018). Development of a complete valorization strategy for *Cistus ladanifer* residues. Congress Iberoamerican Congress on Biorefineries (4-CIAB), 24 - 26 de outubro, Jaén, Espanha.

II. Carvalheiro, F., Duarte, L.C., Moniz, P., Alves-Ferreira, J. (2017). Biomass derived oligosaccharides in the biorefinery context: production and future prospects. 12ª Reunião do Grupo de Glúcidos - GLUPOR12, 11 - 13 de setembro, Aveiro, Portugal.

III. Alves-Ferreira, J., Fernandes, M. C., Duarte, L. C., Martínez, A., Pereira, H., Carvalheiro, F. (2017). "*Cistus ladanifer* as a feedstock for the Mediterranean biorefineries". Workshop of the International Association of Mediterranean Agro-Industrial Waste (IAMAW)". Agro-industrial wastes as profitable sources for bioproducts production, their valorization, Alma Mater Studiorum Università di Bologna, 9 de setembro, Bolonha, Itália.

IV. Alves-Ferreira, J., Fernandes, M. C., Duarte, L. C., Martínez, A., Pereira, H., Carvalheiro, F. (2017). "*Cistus ladanifer* as a feedstock for the Mediterranean biorefineries". 9th International Conference on Environmental Engineering and Management (ICEEM 09), Alma Mater Studiorum Università di Bologna, 6 - 7 de setembro, Bolonha, Itália.

V. Alves-Ferreira, J., Fernandes, M. C., Duarte L. C., Pereira, H., Carvalheiro, F. (2015). "Valorização da esteva num enquadramento de biorefinaria". 10º Seminário de Engenharia das Energias Renováveis e Ambiente, Escola Superior de Tecnologia e Gestão de Portalegre, 27 de maio, Porto Alegre, Portugal.

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I. Alves-Ferreira, J., Fernandes, M. C., Duarte, L. C., Martinez, A., Pereira, H., Carvalheiro, F. (2018). Development of a complete valorization strategy for *Cistus ladanifer* residues. Book of proceedings of 4 - CIAB, 4º Congresso Ibero-americano de Biorrefinarias, 24 - 26 de outubro, Jaén, Espanha.

Painel:

I. Alves-Ferreira, J., Morgado, F., Duarte, L. C., Fernandes, M. C., Roseiro, L. B., Pereira, H., Carvalheiro, F. (2019). Effect of different delinignification processes on *Cistus ladanifer* distillery residues. Jornadas MED - Mediterranean Institute for Agriculture, Environment and Development". Pólo da Mitra, Universidade de Évora, 27 e 28 de Junho, Évora, Portugal.

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III. Alves-Ferreira, J., Fernandes, M. C., Duarte, L. C., Pereira, H., Carvalheiro, F. (2017). *Cistus ladanifer* waste biomass as feedstock for oligosaccharides production. 12ª Reunião do Grupo de Glúcidos - GLUPOR12, 11 -13 de setembro, Aveiro, Portugal.

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V. Alves-Ferreira, J., Duarte, L. C., Fernandes, M. C.; Pereira, H.; Carvalheiro, F. (2015) Hydrothermal treatments of *Cistus ladanifer* residues after essential oil distillation. 3rd Iberoamerican Congress on Biorefineries, 23 – 25 de novembro, Concepcion, Chile.

ABREVIATURAS, SIGLAS E SÍMBOLOS

ABTS	(radical 2,2'azinobis- (3-ethylbenzthiazoline-6-sulfonic acid))
AcOS	Grupos acetilo substituintes dos oligossacarídeos ("acetyl groups linked to oligosaccharides")
AGO	Organosolv com glicerol em meio alcalinizado
AOS	Arabino-oligossacarídeos ("arabino-oligosaccharides")
ASP	Processo aquoso com soda
ATP	Adenosina trifosfato
CAGR	Taxa de crescimento anual composta ("Compound annual growth rate")
CE	Catequina
CEBAL	Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo
CEF	Centro de Estudos Florestais
CL	*Cistus ladanifer* (esteva/"rockrose")
CLR	Resíduos de destilaria de *Cistus ladanifer*
CLRext	Resíduos de destilaria de *Cistus ladanifer* extractados
CLRtreat	Resíduos de destilaria de *Cistus ladanifer* extratados e pré-tratados por auto-hidrólise
CZE	Electroforese capilar ("Capillary Zone Electrophoresis")
D**-LA**	Ácido D-láctico ("D-lactic Acid")
DP	Grau de polimerização
DPPH	Radical 1,1-diphenil-2-picrilhydrazil

EO	Tratamento organosolv usando etanol
EUA	Estados Unidos da América
FPU	Unidade de papel de filtro (“Filter paper unit”)
FRAP	Potencial antioxidante redutor de ferro (“ferric reducing antioxidant potential”)
G	Guaiacilo
GAE	Equivalentes de ácido gálico
GC	Cromatografia gasosa (“Gas chromatography”)
GlcOS	Gluco-oligossacarídeos (“Gluco-oligosaccharides”)
H	*p*-hidroxifenilo
HAA	hidroxiacetaldeído
HMF	5-hidroximetil-2-furaldeído (hidroximetilfurfural)
HPLC	Cromatografia Líquida de Alta Pressão (“High Performance Liquid Chromatography”)
ISA	Instituto Superior de Agronomia
LA	Ácido Láctico (“Lactic Acid”)
LAB	Bactérias lácticas
LB	Luria-Bertani
LHW	“Liquid hot water”

LNEG	Laboratório Nacional de Energia e Geologia
LSR	Razão líquido-sólido (“Liquid-to-Solid Ratio”)
ML	Materiais Lenhocelulósicos
p*K*a	log Ka (Ka = Constante de dissociação ácida)
PLA	Ácido poliláctico (“Polylactic Acid”)
QAH	Hidrólise ácida quantitativa (“Quantitative acid hydrolysis”)
Q_{LA}	Produtividade volumétrica em lactato
RI	Índice de refracção
rpm	Rotações por minuto
S	Siringilo
SHF	Sacarificação e fermentação separada (“Separate Hydrolysis and Fermentation”)
SSF	Sacarificação e fermentação simultânea (“Simultaneous Saccharification and Fermentation”)
SSCF	Sacarificação e co-fermentação simultânea (“Simultaneous saccharification and Co-Fermentation”)
TEAC	Capacidade antioxidante equivalente a Trolox (“Trolox equivalent antioxidant capacity”)
UE	União Europeia

USA	United States of America
UV	Ultravioleta
XOS	Xilo-oligossacarídeos (“Xylo-oligosaccharides”)
Y_{LA}	Rendimento em ácido láctico
μ	Taxa específica de crescimento

INTRODUÇÃO GERAL

Enquadramento

O desenvolvimento da atividade industrial, as flutuações nos mercados dos combustíveis fósseis bem como as exigências em termos ambientais devido às alterações climáticas a nível global, tem conduzido a uma utilização cada vez mais intensiva dos recursos naturais, nomeadamente de recursos biológicos vegetais. Esta utilizaçâo faz-se não só para fins energéticos mas também, e cada vez mais, para a obtenção de bioprodutos.

O aperfeiçoamento da conversão da variedade de recursos vegetais em produtos químicos, energia e materiais é a chave para que a economia mundial volte a basear-se na biomassa sustentável renovável (Liu et al., 2012). Este princípio está ligado à bioeconomia, um novo conceito que entrou oficialmente em discussão no início do século XXI, e que compreende as partes da economia que usam recursos biológicos renováveis da terra e do mar para produzir alimentos, compostos químicos, materiais e energia (European Comission, 2019). Deste modo, aprofundar o conhecimento e investir em sistemas de inovação e transferência de tecnologia com o apoio de políticas públicas são aspectos fundamentais para enfrentar os desafios referentes à valorização desses recursos, em particular, no que diz respeito à utilização de processos eficientes, ambientalmente sutentátveis e de baixo custo.

Neste sentido, a valorização da biomassa no âmbito do conceito de biorrefinaria, tem vindo a ganhar uma relevância crescente, uma vez que uma biorrefinaria integra processos de conversão de biomassa para a obtenção de energia, materiais e produtos químicos, nomeadamente de valor acrescentado (Carvalheiro et al., 2008; SIADEB, 2011), como representado esquematicamente na figura 1. Este conceito desenvolveu-se inicialmente, associado à valorização da biomassa para fins energéticos, sendo uma evolução das unidades industriais de produção de etanol (biorrefinarias de 1ª geração). No entanto, embora as biorrefinarias encontrem-se em grande expansão mundial, necessitam ainda de desenvolvimentos a nível científico e tecnológico para superar as limitações com que atualmente se deparam e para que possam responder ao desafio que se lhes coloca: serem o principal motor do desenvolvimento da bioeconomia, tornando-se unidades industriais geradoras de emprego e riqueza (SIADEB, 2011).

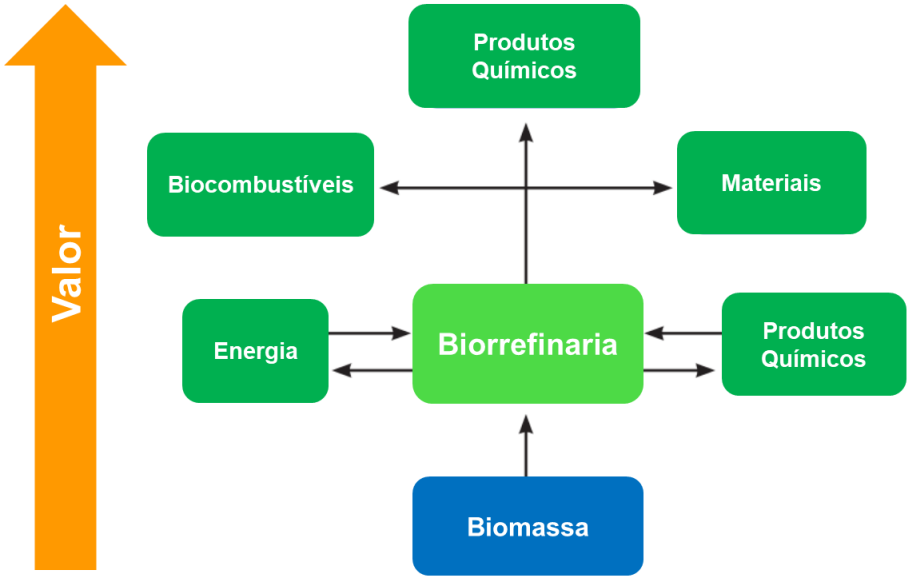

Fig. 1 Esquema simplificado sobre o conceito de uma biorrefinaria (Adaptado de siadeb.org/pt)

Existem vários produtos que podem ser obtidos numa biorrefinaria, tais como, biocombustíveis, alimentos, rações, energia, produtos químicos; dentre estes, os ácidos orgânicos, bem como os compostos bioativos são bastante relevantes. Os compostos fenólicos são provenientes da fracção de extrativos e da lenhina e, devido às suas atividades funcionais, apresentam forte potencial de aplicação em várias indústrias, como a cosmética, farmacêutica e alimentar. Os oligossacarídeos, em especial os xilo-oligossacarídeos, produtos funcionais que podem derivar da hidrólise das hemiceluloses, têm sido amplamente utilizados como ingredientes alimentares (edulcorante, agente de controlo de peso, humectantes, etc.) e suplementos farmacológicos (prebiótico, agente anticariogénico, imunoestimulador, antioxidante, alternativa antibiótica, reguladores da glicemia, etc) (Patel & Goyal, 2011). Para além das aplicações farmacológicas, os oligossacarídeos encontram utilização na indústria cosmética, alimentação animal e pesqueira, agricultura, etc (Patel & Goyal, 2011).

Relativamente aos ácidos orgânicos, destaca-se o ácido láctico, utilizado para produzir PLA (ácido poliláctico) que, por sua vez, é utilizado no fabrico de bioplásticos (substituto de plásticos derivados do petróleo). A capacidade global de produção de bioplásticos em 2023 deverá aumentar cerca de 25% em relação a 2018 (European Bioplasctics, 2018).O ácido láctico também apresenta diversas aplicações nas indústrias alimentar, cosmética, médica e farmacêutica (Martinez et al., 2013), cujo mercado global deverá alcançar 8,77 mil milhões de dólares até 2025 (Grand View Research, 2018). O ácido láctico pode ser obtido a partir da fermentação dos açúcares extraídos de fontes

renováveis, como cana-de-açúcar e milho, mas também e, desejavelmente, a partir de resíduos e subprodutos lenhocelulósicos que são mais favoráveis do ponto de vista da sustentabilidade. Contudo, é de salientar que as tecnologias de produção de ácido láctico precisam necessitam de maiores avanços para se tornarem técnica e economicamente viáveis e ambientalmente sustentáveis. Para tal, o uso de resíduos lenhocelulósicos abriu uma via de trabalho importante para se agregar valor com base numa tecnologia ecologicamente amigável (John et al., 2007).

Entre os resíduos lenhocelulósicos mais abundantes encontra-se o bagaço de cana-de açúcar, os resíduos do processamento do milho, a palha de trigo e da casca de arroz e os resíduos florestais. Estes últimos incluem os resíduos gerados nas florestas (bicadas, folhas, ramos e casca), os resíduos provenientes das indústrias da pasta para papel e de transformação de madeiras e os resíduos provenientes de diferentes espécies herbáceas ou arbustos (matos). Nalguns casos, trata-se de espécies infestantes ou que se desenvolvem com grande abundância, podendo constituir um problema ambiental, mas que podem ser utilizados como materiais alternativos e para os quais urge encontrar uma valorização. O presente estudo consiste na utilização de materiais lenhocelulósicos de espécies arbustivas espontâneas como matérias-primas para biorrefinarias. A esteva (*Cistus ladanifer*), existente em todo o território nacional português e na zona mediterrânica, é um exemplo típico dessas espécies e foi usada como planta modelo neste trabalho. *Cistus ladanifer* (CL) é utilizada atualmente para a produção de óleos essenciais e lábdano, produtos estes que encontram grande aplicação comercial para a indústria da cosmética e perfumaria (Marioti et al., 1997; Gomes et al., 2005; Teixeira et al., 2007). Acresce que os resíduos provenientes das destilarias de óleos essenciais são materiais ricos em polissacarídeos e lenhina e com uma fração importante de extrativos, entretanto não se conhecem outras utilizações para estes resíduos a não ser a energética (combustão) e a compostagem. Portanto, a valorização da biomassa remanescente da destilação para produção de óleos essenciais permitiria melhorar a rentabilidade económica dessa planta aromática permitindo não somente a obtenção de óleos essenciais, mas também a produção de uma gama alargada de co-produtos a partir da fração extrativa e lenhocelulósica.

Este trabalho inclui estudos sobre o fracionamento seletivo dos componentes da biomassa vegetal (celulose, hemiceluloses e lenhina), cuja desconstrução da matriz celular permite o desenvolvimento de bioprodutos de origem lenhocelulósica, bem como a separação de extrativos como compostos bioativos.

Assim, foram desenvolvidos processos integrados para o fracionamento da biomassa que incluíram (Fig. 2): 1) a separação sequencial de extrativos por meio de um sistema Soxhlet em escala piloto, sendo utilizados o etanol e a água como solventes; 2) o

pré-tratamento da biomassa extratada recorrendo-se ao processo de auto-hidrólise que permite a recuperação elevada de hemiceluloses e preservação da celulose e da lenhina; 3) estudo de diferentes processos de deslenhificação para a recuperação dos compostos fenólicos derivados de lenhina, bem como para o aumento da digestibilidade enzimática da celulose remanescente. Dessa forma, a biomassa pré-tratada hidrotermicamente foi submetida a dois processos organosolv: misturas etanol/água e glicerol/água em meio alcalino, sendo estes comparados com o tratamento alcalino, utilizando hidróxido de sódio; e 4) fermentação dos açúcares celulósicos e hemicelulósicos obtidos nos processos de pré-tratamento (hidrólise das hemiceluloses e deslenhificação) para a produção de ácido D-láctico utilizando uma estirpe de *Escherichia coli* transformada (JU15) (Utrilla et al., 2012). A utilização dessa estirpe com a capacidade de produzir eficientemente D-lactato a partir de pentoses e hexoses constituiu um dos aspectos mais inovadores deste trabalho.

Fig. 2 Esquema dos processos integrados utilizados para a valorização dos resíduos de destilarias da esteva

Dessa forma, o desenvolvimento e a otimização de processos integrados para a utilização de recursos florestais num quadro de biorrefinaria é particularmente relevante para a valorização de biomassa endógena com grande potencial de exploração em pequena e grande escala.

Objetivos

Este trabalho teve como objetivo principal estudar e propor uma abordagem metodológica para a bioconversão de hexoses e pentoses, provenientes da celulose e hemiceluloses, para a produção de ácido D-láctico e para a obtenção de novos bioprodutos derivados dos extrativos e da lenhina, usando como fonte de biomassa os resíduos de *Cistus ladanifer* disponíveis após a destilação para extração de óleos essenciais .

O estudo mais detalhado da composição química de *Cistus ladanifer* e a otimização de processos de fracionamento e fermentação perspectivaram um novo uso para os resíduos de destilaria desta espécie. Neste contexto, o trabalho consistiu principalmente nos seguintes objetivos específicos:

i. Determinar a composição química e características estruturais de *C. ladanifer* e dos seus resíduos obtidos a partir da destilação de óleos essenciais, a fim de fornecer dados de base para a sua integração em biorrefinarias;

ii. Estudar o teor e composição dos extrativos solubilizados em diferentes solventes, bem como identificar e avaliar as atividades biológicas de compostos fenólicos indicando o potencial desses extratos como produtos de valor acrescentado;

iii. Otimizar o processo de auto-hidrólise a fim de melhorar os rendimentos de xilo-oligossacarídeos (XOS) e açúcares solúveis em geral, minimizando a concentração de inibidores e obter sólidos enriquecidos em celulose e lenhina;

iv. Avaliar e comparar a eficiência de diferentes métodos para a deslenhificação dos sólidos pré-tratados, nomeadamente tecnologias organosolv (misturas etanol/água e glicerol/água em meio alcalino) e tratamentos alcalinos;

v. Estudar diferentes modos de fermentação utilizando os hidrolisados hemicelulósicos provenientes do pré-tratamento hidrotérmico e os sólidos enriquecidos em celulose, usando a estirpe geneticamente modificada *E.coli* JU15 para o desenvolvimento de um processo eficiente de produção de ácido D-láctico.

Estrutura da Tese

Esta tese está organizada em oito capítulos que incluem uma revisão bibliográfica (**Capítulo I**) que aborda os principais conceitos teóricos que fundamentaram o trabalho experimental, seguido por um artigo de revisão (**Capítulo II**) que apresenta os conhecimentos gerais disponíveis sobre a esteva, tendo em conta o seu potencial como planta modelo a ser usada num quadro de biorrefinaria. Os **Capítulos III a VII** estão

apresentados também na forma de artigos, todos já publicados ou submetidos em revistas internacionais com arbitragem científica, e que estão ordenados de acordo com os experiências sequenciais que delinearam esta pesquisa: i) preparação e caracterização da matéria-prima; ii) fraccionamento selectivo de hemiceluloses utilizando pré-tratamento hidrotérmico; iii) estudos de processos de deslenhificação; iv) desenvolvimento da produção de ácido láctico.

Mais detalhadamente, o **capítulo I** aborda o estado dos conhecimentos referentes aos temas tratados nesta tese. Neste capítulo são sumariamente descritos o conceito e os aspectos gerais de uma biorrefinaria e, neste âmbito, a importância da valorização dos materiais lenhocelulósicos para obtenção de produtos de valor acrescentado a partir dos seus constituintes. Apresentam-se as principais particularidades referentes ao fracionamento seletivo da matéria-prima lenhocelulósica, com destaque para os processos de auto-hidrólise e processos alcalinos e organosolv. São abordados também os efeitos dos compostos inibidores presentes em licores hemicelulósicos utilizados em processos fermentativos e os métodos de destoxificação mais usuais para a remoção destes compostos. Por fim, são destacadas as principais informações a respeito da produção do ácido láctico e suas possíveis aplicações, bem como os diferentes modos de fermentação e microrganismos fermentadores, com particular destaque para a bactéria *Escherichia coli* transformada (JU15).

O **Capítulo II** apresenta uma pequena revisão sobre a potencial utilização da esteva como matéria-prima a ser utilizada num contexto de biorrefinaria. Em vista disso, são expostas as informações gerais sobre as suas principais características morfológicas e comportamentais e sobre os diferentes produtos obtidos a partir dos processos de destilação e extração, destacando as suas propriedades funcionais e utilizações. São também discutidas as perspetivas futuras para o uso da esteva enquanto matéria-prima lenhocelulósica e a janela de oportunidades que se poderá abrir face à valorização integrada dos seus resíduos para a obtenção de produtos de valor acrescentado.

No **Capítulo III** avalia-se a caracterização química e estrutural detalhada da esteva, tendo como base as diferentes partes morfológicas de plantas recolhidas diretamente do campo e da biomassa residual proveniente de destilarias de óleos essenciais. Em síntese, são apresentadas a composição química somativa, a composição monomérica dos polissacarídeos e da lenhina, a avaliação da composição e propriedades antioxidantes dos extrativos assim como aspectos estruturais do caule, incluindo biometria celular, utilizando técnicas de microscopia ótica.

Nos **Capítulos IV e V** são estudados a aplicação de processos de pré-tratamento hidrotérmico (auto-hidrólise) para o fracionamento das hemiceluloses, utilizando biomassa não extratada e extratada, respectivamente. A utilização da

biomassa contendo extrativos permitiu avaliar os efeitos da auto-hidrólise para a remoção destes compostos e o seu impacto nos rendimentos dos açúcares. Neste âmbito e considerando as concentrações e o potencial valor desses extrativos, efetuou-se a sua remoção (à escala piloto) e condução de uma nova otimização em material extratado. Dessa forma, foi possível comparar as duas abordagens quanto à solubilização dos açúcares, formação de subprodutos e composição da fração sólida obtida, e, por conseguinte, apresentar a direção mais viável para o fracionamento não somente das hemiceluloses, mas também dos extrativos.

No **Capítulo VI** são comparados diferentes processos de deslenhificação utilizando as amostras extratadas resultantes da condição otimizada no processamento hidrotérmico. Os processos de deslenhificação foram conduzidos com o propósito de remover a quantidade máxima de lenhina e a máxima recuperação de compostos fenólicos e sólidos ricos em celulose. Neste estudo foram usados processos com solventes (organosolv) e alcalinos, sob diferentes condições operacionais (temperatura/ tempo/catalisador). Salienta-se que no processo organosolv foram estudadas diferentes abordagens com base na utilização de misturas etanol/água ou misturas glicerol/água em meio alcalinizado com hidróxido de sódio (NaOH). No processo alcalino também foram utilizadas soluções de NaOH com várias concentrações. Para uma melhor avaliação química e estrutural dos sólidos ricos em celulose e das lenhinas solubilizadas, efetuaram-se análises complementares por meio de digestibilidade enzimática e pirólise analítica.

O **Capítulo VII**, corresponde ao desenvolvimento do processo de produção de ácido D-láctico. Este estudo consistiu, principalmente, na realização de ensaios de fermentação dos hidrolisados e dos sólidos obtidos durante os processos de fracionamento, usando a bactéria transformada *E. coli* JU15. Os licores hemicelulósicos foram inicialmente submetidos a ensaios de hidrólise ácida diluída para a obtenção de um rendimento elevado de açúcares fermentescíveis, preconizando ao mesmo tempo a economia com o catalisador e tempo de reação. Num passo subsequente, os hidrolisados foram sujeitos a processos de destoxificação para comparação de desempenho fermentativo em relação aos hidrolisados não destoxificados. A produção de ácido láctico efetuou-se recorrendo-se a diferentes modos de fermentação: fermentação dos hidrolisados destoxificados e não destoxificados; hidrólise enzimática e fermentação separadas (SHF); pré-sacarificação e fermentação simultâneas (SSF); pré-sacarificação e co-fermentação simultâneas (SSCF) dos sólidos ricos em celulose em combinação com os hidrolisados hemicelulósicos destoxificados. Neste capítulo abordam-se as vantagens de cada um dos modos de fermentação visando a economia global do processo sob o conceito de uma estratégia de valorização completa da matéria-prima.

No **Capítulo VIII,** são enunciadas as principais conclusões obtidas com a realização deste trabalho e as perspectivas gerais para futuras investigações.

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CAPÍTULO I

Revisão Bibliográfica

1. Considerações gerais sobre Biorrefinarias

O conceito de biorrefinaria tem por base a valorização eficiente da biomassa para a produção concomitante de biocombustíveis e produtos de valor acrescentado de uma forma económica, social e ambientalmente sustentável. O termo não se aplica apenas a uma indústria ou sistema de processo específico, mas abrange uma ampla gama de sistemas técnicos, uma vez que a biorrefinaria pode ser uma única máquina para a conversão de biomassa até uma complexa instalação industrial de poligeração integrada a outras indústrias e sistemas de energia, onde diferentes tipos de matéria-primas são refinadas em um ou muitos produtos usando transformações químicas, bioquímicas e termoquímicas (Tsagaraki et al., 2017). A ampla gama de tecnologias utilizada numa biorrefinaria é capaz de separar os diferentes recursos de biomassa (madeira, gramíneas, milho, etc) nos seus blocos de construção (hidratos de carbono, lenhina, proteínas, triglicéridos, compostos fenólicos, etc.) que, por sua vez, podem ser convertidos em produtos de valor acrescentado (Cherubini, 2010) (Fig. 1).

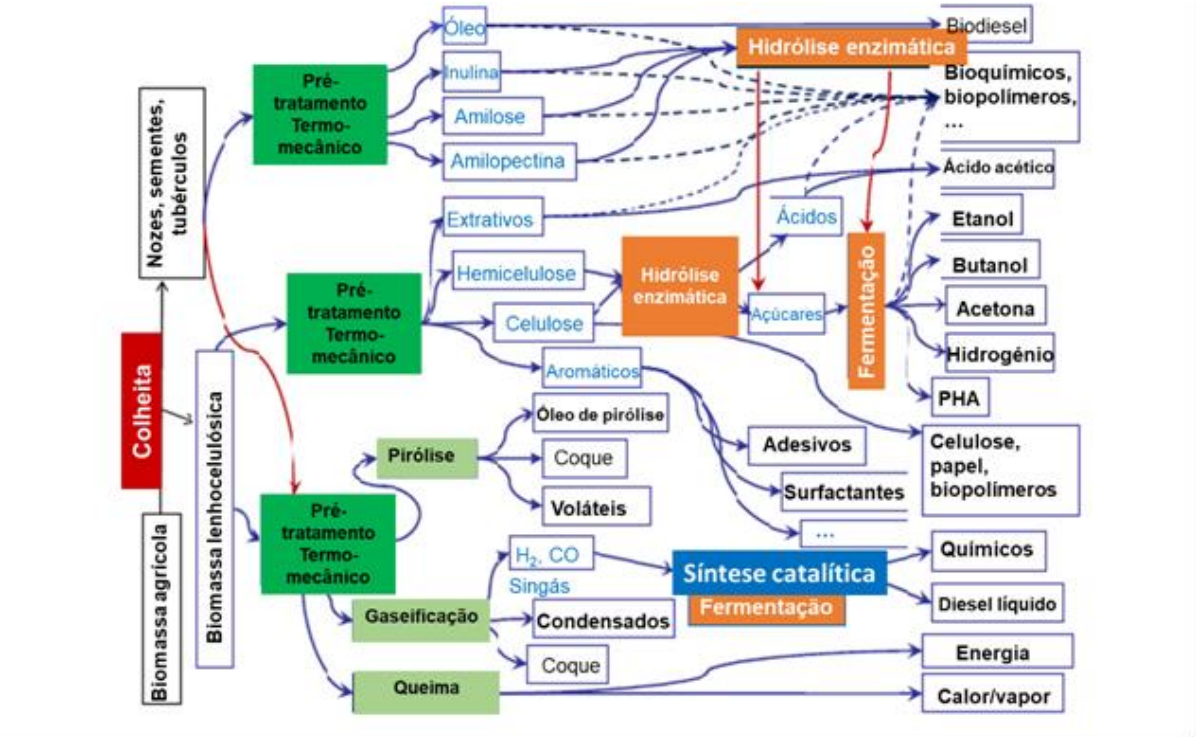

Fig. 1 Esquema de processos aplicados para a obtenção de produtos em biorrefinaria (Adaptado de Liu et al., 2012a)

O conceito de biorrefinaria é análogo ao da refinaria de petróleo uma vez que, similarmente ao petróleo, a composição complexa da biomassa exige a sua separação primária em grupos de substâncias principais, bem como o seu tratamento e

processamento para a obtenção de produtos (Kamm et al., 2006). Todavia, enquanto as refinarias de petróleo surgiram com base na engenharia química, as biorrefinarias nascem da interface entre a engenharia química e a engenharia biológica (Bastos, 2012). Quanto aos produtos, as biorrefinarias, à semelhança das refinarias de petróleo, também produzem uma combinação de vários produtos com uma pegada ambiental potencialmente menor (Fernando et al, 2006; Bastos, 2012).

Alguns compostos como ácidos orgânicos (ácidos succínico, fumárico, málico, glutárico, levulínico, láctico), aminoácidos (ácidos aspártico e glutâmico) e polióis (glicerol, sorbitol e xilitol/arabitol) estão entre os produtos preferenciais a serem obtidos a partir dos açúcares da biomassa lenhocelulósica por via bioquímica (Werpy & Petersen, 2004), pois podem ser possíveis Building blocks (precursores) para a indústria química. A lenhina também tem sido avaliada quanto às suas potencialidades no enquadramento das biorrefinarias. As principais oportunidades para esse polímero podem ser agrupadas em 3 categorias de produtos: biocombustíveis e gás de síntese, macromoléculas, e compostos aromáticos monoméricos (Holladay et al., 2007).

Dado todo este potencial, as principais barreiras e desafios a serem superados para a criação de biorrefinarias são (Kretschmer et al., 2013):

- ✓ disponibilidade fiável e preços competitivos para a biomassa;
- ✓ tecnologias comprovadas à escala comercial, cruzando a lacuna de inovação entre a escala de demonstração e plena comercialização;
- ✓ financiamento adequado para a criação de unidades de demonstração em grande escala (comercial) ou das primeiras instalações do género;
- ✓ demanda suficiente do mercado para facilitar os investimentos e dar o passo para a comercialização;
- ✓ um quadro político de longo prazo previsível e estável, e o apoio público para a utilização de biomassa no setor da energia.

Várias empresas ao redor do mundo estão instalando tecnologias de ponta para produzirem biocombustíveis e bioprodutos a partir da biomassa lenhocelulósica. Alguns investimentos importantes vêm sendo feitos pelos Estados Unidos da América (EUA) e pela União Europeia (UE), por meio de financiamentos governamentais ou de investidores, na implantação de unidades de demonstração pré-comercais, favorecendo a ampliação de novas tecnologias (Balan et al., 2013). Muitos desses projetos industriais, adotaram a plataforma de conversão bioquímica para a produção de bioetanol, onde são usados métodos de pré-tratamento da biomassa seguidos pelo processo de fermentação. Outras unidades baseam-se na plataforma termoquímica que utiliza processos com

temperaturas entre 300 ºC e 1000 ºC, com pouco ou nenhum oxigénio (Azapagic, 2014) tais como a pirólise, liquefação e gaseificação para produzirem hidrocarbonetos líquidos de cadeia longa. As unidades de plataformas integradas (isto é, termoquímicas e bioquímicas combinadas) também têm sido implementadas, especialmente nos EUA, para produzirem tanto hidrocarbonetos líquidos de cadeia longa como bioetanol (Balan et al., 2013). Uma biorrefinaria integrada torna-se interessante e desejável, considerando que a combinação das várias tecnologias de conversão podem reduzir o custo total e proporcionar mais flexibilidade na geração de produtos e no fornecimento de sua própria energia (Fernando et al., 2006).

Em termos globais, a conversão de recursos biológicos em produtos de valor acrescentado, bem como as vias de inovação baseadas nesses recursos apresentam potencial considerável e devem ser desenvolvidos. No entanto, garantir a transparência e informação sobre as disponibilidades dos fluxos da biomassa, as oportunidades de processamento e os benefícios para os consumidores são pontos importantes para encorajar possíveis investidores (Kretschmer et al., 2013), a par dos desafios de sustentabilidade ambientais, econômicos e sociais. Estes devem ser considerados tendo por base todo o ciclo de vida de um sistema de biorrefinarias, desde onde este se inicia, no cultivo e colheita da biomassa (se aplicável), passando pela sua armazenagem e transporte, conversão para os diversos produtos, até ao consumo destes pelos utilizadores. Isso é necessário para evitar a mudança dos impactos da sustentabilidade de uma parte da cadeia de fornecimento para outra, por exemplo, reduzindo as emissões de gases de efeito estufa da refinaria apenas para aumentá-las por meio do transporte de matérias-primas (Azapagic, 2014).

2. Composição da biomassa lenhocelulósica

A consciência social dos impactos ambientais causados pelos combustíveis fósseis, bem como os problemas de estabilidade e sustentabilidade do fornecimento de energia destas fontes convergiram para criar uma necessidade de desenvolver sistemas energéticos mais sustentáveis baseados em biomassa lenhocelulósica (Agbor et al., 2011; Liu et al., 2012a). Dada a sua natureza renovável e a baixa poluição associada, os materiais lenhocelulósicos são considerados uma fonte importante para a produção de combustíveis, pasta para papel, biomateriais e uma variedade de produtos químicos (Vallejos et al., 2017).

Uma das limitações na utilização desses materiais reside na sua natureza heterogénea e composição complexa. Os materiais lenhocelulósicos consistem em três frações químicas estruturais, celulose, hemiceluloses e lenhina (Quadro 1), de componentes não-estruturais (extrativos, cinza, e outros componentes minoritários) e ainda de outras frações existentes em menores quantidades (por exemplo, pectinas, proteínas, gomas, resinas, amido, etc.) que podem ser importantes em substratos específicos (Gullón et al., 2012 ; Area & Popa, 2014).

Esta estrutura complexa e altamente ordenada torna a biomassa lenhocelulósica fortemente recalcitrante à degradação, enquanto a heterogeneidade na composição e estrutura molecular dos seus componentes contribuem para as diferenças nas propriedades físicas e químicas entre diferentes espécies (Klamrassamee et al., 2013). A Figura 2 mostra uma representação gráfica da biomassa lenhocelulósica com os principais constituintes da parede celular.

Fig. 2 Representação artística da parede celular com fibras de celulose (verde), hemiceluloses (verde claro) e lenhina (castanho) (Fonte: http://portfolio.scistyle.com/Lignocellulose)

Quadro 1. Composição química de diferentes tipos de biomassa lenhocelulósica (% em base seca; Adaptado de Carvalheiro, 2005)

Material	Celulose	Hemiceluloses	Lenhina	Referência
Madeiras resinosas				
Abeto	43.51,1	15,2-26	27,3-26	Fengel e Wegener (1984); Olsson e Hähn-Hagerdal (1996)
Pseudotsuga	43	23	28	Mabee et al. (2006)
Madeiras folhosas				
Amieiro	40,5	18,4	20,8	Taherzadeh et al. (1997)
Carvalho	38,4-44	18,7-23,8	21,5-24,7	Kim et al. (2000)
Choupo	39-51,3	21-28,4	20,3-26	Mok & Antal (1992); Capek-Ménard et al. (1987)
Eucalipto	38-54,0	15-30	23,1-37	Carrasco et al. 1986; Miranda & Pereira 2002
Plátano	40	23	22	Mok & Antal (1992)
Vidoeiro	40,7	22,4	19,1	Taherzadeh et al. (1997)
Materiais agrícolas e agro-industriais				
Bagaço de azeitona	36,4	26,8	26	Neureiter et al. (2002)
Bagaço de cana-de-açúcar	42	25	20	Kumar & Sharma (2017)
Cana-de-açúcar energética	37	18	15	Mok & Antal (1992)
Carolo de milho	45	35	15	Kumar & Sharma (2017)
Casca de amêndoa	30,2	28,5	27,4	Nabarlatz et al. (2007)
Cascas de arroz	36,7-37,7	16,7-17,3	21,3-22,1	Sasaki et al. (2003); Cruz et al. (2001)
Casca de nozes	25 - 30	25 – 30	30 - 40	Kumar & Sharma (2017)
Palha de arroz	36,9-43,4	19,0-22,6	9,9-17,2	Lee (1997); Roberto et al. (2003)
Palha de cevada	33	22,3	16,1	García-Aparicio et al. (2006)
Palha de trigo	29 - 35	26 – 32	16 - 21	Kumar & Sharma (2017)
Sorgo	36-45	18-27	16-20	Mok & Antal (1992); Kumar & Sharma (2017)

2.1. Principais componentes da biomassa lenhocelulósica e aplicações

2.1.2. Celulose

A celulose é a matéria-prima orgânica mais abundante no mundo. Em geral, o seu teor varia de 40-50% na madeira, em torno de 20% nalgumas gramíneas, podendo alcançar até 90% nas fibras de algodão (Bayer & Lamed, 1992). Também pode estar presente em bactérias, fungos e algas (Agbor et al., 2011).

A celulose é um polímero notavelmente estável devido à sua estrutura terciária, consistindo num arranjo linear formado por unidades de β-D-glucopiranose unidas por ligações β- (1,4) glicosídicas (Bayer & Lamed, 1992; Agbor et al., 2011; Quiroz-Castañeda & Folch-Mallol, 2013) (Fig. 3). O grau de polimerização da celulose (DP), isto é, o número de unidades monoméricas em uma molécula do polímero (na celulose essa unidade é a glucose) pode variar de 100 a 20000, dependendo do material (Quiroz-Castañeda & Folch-Mallol, 2013; Biswas & Ahring, 2016).

A celulose forma as microfibrilas que são o elemento estrutural central nas paredes de quase todas as células vegetais e estão altamente organizadas através de ligações de hidrogênio inter e intramoleculares e forças de Van der Waals (Quiroz-Castañeda & Folch-Mallol, 2013; Williamson et al., 2002). Individualmente, a ligação de hidrogênio é bastante fraca, mas coletivamente resulta em uma força associativa muito resistente (Bayer & Lamed, 1992). As microfibrilas agregam-se ordenadamente constituindo áreas cristalinas, que representam cerca de 75% da fibra de celulose (Bayer & Lamed, 1992). As regiões onde as ligações de hidrogénio são quebradas e esse arranjo ordenado é perdido compreendem as áreas amorfas (Quiroz-Castañeda & Folch-Mallol, 2013).

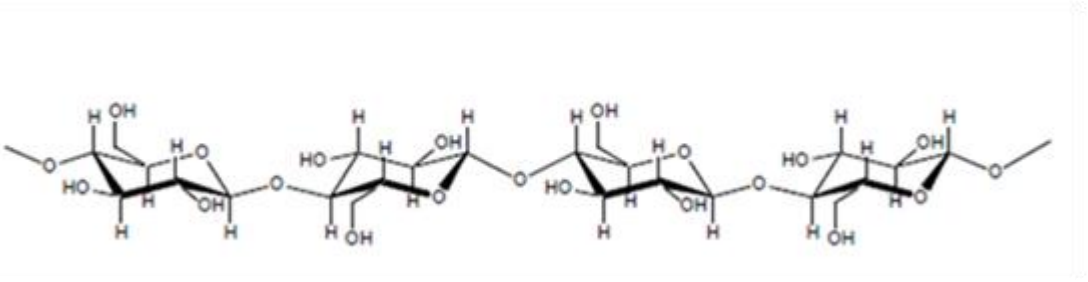

Fig. 3 Representação da estrutura química da celulose (Adaptado de Pereira et al., 2003)

A pasta celulósica, para além da utilização tradicional no fabrico de papel, é atalmente usada em vários mercados de alto valor. As fibras, éteres, ésteres e nitrocelulose são produtos celulósicos que apresentam maturidade comercial e podem ser utilizados em várias aplicações como, têxteis, vestuário, embalagens, produtos de

higiene, produtos farmacêuticos, detergentes, cosméticos, pintura, explosivos, produtos alimentares, filmes, moldagem, entre outros (Kretschmer et al., 2013). A produção de acetato de celulose, por exemplo, tem um mercado bem estabelecido atingindo cerca de 700000 toneladas por ano (Tsagaraki et al., 2017).

A nanocellulose, que se refere aos materiais celulósicos com dimensões estruturais definidas (nanocristais de cellulose, nanofibras de celulose ou nanocelulose bacteriana), devido às suas propriedades mecânicas, ópticas, de resistência, de biocompatibilidade, entre outras, tem encontrado diversas aplicações em variados setores como o automóvel (janelas de automóveis), de construção (compósitos estruturais), eletrónico (membrana para dispositivo eletroacústico), farmacéutico (medicamentos, implantes, stents), de embalagem e papel (embalagens especiais para alimentos) e cosmético (Sharma et al., 2019).

A celulose,tal como as hemiceluloses podem ser fracionadas por meio de métodos químicos ou enzimáticos para produzir açúcares simples que podem ser convertidos a combustíveis (por exemplo, etanol, butanol, acetona, isobutanol, lipídeos, etc) ou outros produtos (ácidos orgânicos) usando fungos, leveduras ou bactérias (Kretschmer et al., 2013; Balan, 2014). Entretanto, em comparação com as hemiceluloses, a celulose é mais resistente a ataques químicos, térmicos e biológicos (Liu et al., 2012a). De facto, alguns microrganismos especializados na degradação da parede celular das plantas podem hidrolisar a celulose. Entre estes, estão incluídos alguns fungos e bactérias anaeróbias e aeróbias (Quiroz-Castañeda & Folch-Mallol, 2013). Porém, os tipos e número de microrganismos com capacidade de degradar eficientemente ou solubilizar a celulose é escasso (Bayer & Lamed, 1992). Esta recalcitrância dos materiais celulósicos à hidrólise para produzir açúcares fermentescíveis é um dos desafios tecnológicos mais importantes e difíceis de superar (Quiroz-Castañeda & Folch-Mallol, 2013).

2.1.2. Hemiceluloses

As hemiceluloses, ao contrário da celulose, não contêm apenas unidades de glucose, mas são compostas de diferentes monossacarídeos de pentoses e hexoses. As hemiceluloses tendem a apresentar uma massa molecular inferior à da celulose, e a estrutura molecular é levemente ramificada (Demirbas, 2004). O grau de polimerização das hemiceluloses é bastante inferior ao da celulose, variando de 50 a 300 (Biswas & Ahring, 2016). Como resultado de seu DP mais baixo, as hemiceluloses apresentam

menor estabilidade química e térmica e, consequentemente, são mais solúveis e suscetíveis à hidrólise do que a celulose (Ajao et al., 2018).

Os monossacarídeos encontrados nas hemiceluloses incluem pentoses (β-D-xilose, α-L-arabinose), hexoses (β-D-manose, β-D-glucose, α-D-galactose) e ácidos urónicos (ácido β-D-glucurónico, ácido α-D-4-O-metilglucurónico e ácido α-D-galacturônico) (Fig 4). Entretanto, α-L-ramnose e α-L-fucose podem estar presentes em pequenas quantidades (Pereira et al., 2003). Os grupos OH dos açúcares podem ser parcialmente substituídos com grupos acetilo (Carvalheiro, 2005). As hemiceluloses estão ligadas principalmente por ligações glicosídicas β-1,4, mas também podem ser encontradas as ligações glicosídicas β-1,3-; β-1,6; α-1,2; α-1,3 e α-1,6 (Área & Popa 2014).

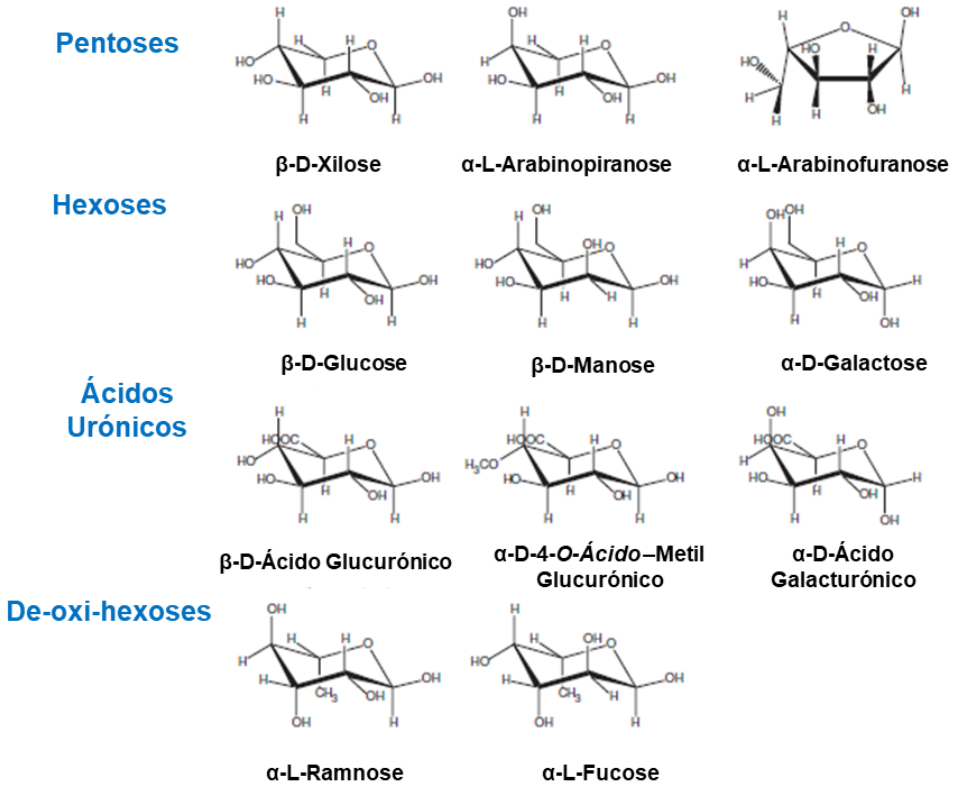

Fig. 4 Fórmulas estruturais das principais unidades monossacarídicas em hemiceluloses (Adaptado de Pereira et al., 2003)

A classificação dos polimeros presentes nas hemicelluloses, é feita de acordo com o açúcar predominante na cadeia principal e na ramificação lateral. Desta forma, as xilanas são formadas por cadeias principais de unidades de D-xilose, enquanto as mananas são compostas de D-manose e as xiloglucanas de D-glicose, contendo unidades de D-xilose (Reiter, 2002 ; Alvarez et al., 2016).

A distribuição de hemiceluloses varia de acordo com o tipo de matéria-prima (Pereira et al., 2003). A principal diferença entre as espécies folhosas e resinosas, por exemplo, é a distribuição da fração hemicelulósica entre hexoses e pentoses. As folhosas são principalmente constituídas por xilose e grupos arabinose (C5), enquanto as resinosas têm uma fração maior de galactose, manose e glucose (C6) (Ajao et al., 2018). Como resultado dessas diferenças na composição, as espécies folhosas são menos recalcitrantes que as resinosas à ação de enzimas (Álvarez et al., 2016). Já nas plantas anuais, como milho, arroz, aveia, girassol, cevada são predominantes as arabinoxilanas (Ren and Sun, 2010). Em geral, estas últimas são estruturalmente mais diversificadas e complexas. Apresentam uma cadeia principal de β-D-xilopiranosil que pode ser fortemente ramificada com xilopiranose, arabinofuranose e galactopiranose (Ren & Sun, 2010).

As hemiceluloses são solúveis em soluções alcalinas, contudo, as glucomananas com um elevado teor de galactose, e as arabinogalactanas altamente ramificadas são exemplos de hemiceluloses solúveis em água (Pereira et al., 2003). Dessa forma, os tratamentos aquosos à temperaturas na gama 160-220 °C são adequados para a separação das hemiceluloses dos outros constituintes poliméricos (Gullón et al., 2012). O método de extração de hemiceluloses também desempenha um papel importante na escolha do processo para a transformação dos açúcares. Por exemplo, um método de extração que pode decompor os açúcares oligoméricos em monómeros não exigirá uma hidrólise subsequente com ácido ou enzimas, mas também podem ter algumas desvantagens, como a degradação concomitante da celulose e a sua qualidade (Ajao et al., 2018).

Os açúcares derivados de hemiceluloses podem ser usados diretamente ou convertidos em biocombustíveis, ácidos orgânicos, álcoois de açúcar ou furanos. A transformação dos açúcares pode ser por meio de conversão bioquímica (fermentação) ou química. Algumas das utilizações diretas dos açúcares hemicelulósicos incluem o cultivo de leveduras, a produção de enzimas e filmes de polímeros para embalagem (Ajao et al., 2018).

Oligossacarídeos

Os oligossacarídeos (OS) são provenientes de várias fontes, como bactérias, algas, fungos e plantas superiores, e têm sido amplamente utilizados como ingredientes alimentares e suplementos farmacológicos. Os oligossacarídeos funcionais estão entre os polissacarídeos não digeríveis, ou seja, aqueles que não são digeridos no estômago e

atingem o intestino grosso de forma intacta (Gullón et al., 2010) e apresentam destaque e popularidade devido aos seus benefícios fisiológicos para os consumidores. Entre as muitas propriedades fisico-químicas e fisiológicas importantes dos oligossacarídeos não digeríveis destacam-se a sua atuação como prebióticos para melhorar a microecologia e imunomodulação do intestino, bem como o fornecimento de proteção contra a adesão de patógenos (Wang et al., 2018). Eles têm sido comercializados desde a década de 1980 como agentes de baixo teor calórico e são também usados como humectantes por causa de sua alta capacidade de retenção de humidade (Patel & Goyal, 2011).

Os oligossacarídeos apresentam baixo peso molecular e são de natureza intermediária entre os açúcares simples e os polissacarídeos e, em geral, apresentam graus de polimerização (DP) entre 3 e 10 (Weijers et al. 2008). No entanto, as moléculas com DP entre 11 a 19, também têm sido reconhecidas como oligossacarídeos devido às suas propriedades similares (Wang et al., 2018). Os pesos moleculares mais altos em comparação com as soluções de mono e dissacarídeos, produzem soluções mais viscosas. A sua estabilidade depende do teor de resíduos do açúcar, da forma de anel, da configuração anomérica e do tipo de ligação (ligações β mais são fortes que as ligações-α e as hexoses são mais fortemente ligadas do que as pentoses) (Patel & Goyal, 2011).

As principais categorias de oligossacarídeos, atualmente disponíveis ou em desenvolvimento como ingredientes alimentares, incluem compostos nos quais a unidade monossacarídica é frutose, galactose, glucose e/ou xilose (Mussatto & Mancilha, 2007). Algumas dessas classes são comercializadas como agentes bifidogénicos, tais como: ciclodextrinas, fruto-oligossacarídeos, galacto-oligossacarídeos, isomaltulose, rafinose, gentio-oligossacarídeos, glicosil sacarose, isomalto-oligossacarídeos, lactosucrose, lactulose, malto-oligossacarídeos, oligossacarídeos de soja, xilo-oligossacarídeos (Sako et al., 1999).

Os oligassacarídeos hemicelulósicos, nomeadamente os xilo-oligossacarídeos (XOS), obtidos a partir da hidrólise das xilanas, possuem grande potencial prebiótico e podem ser usados como compostos bioativos em muitos produtos alimentares (Aachary & Prapula, 2010). O caráter prebiótico dos XOS está relacionado principalmente com a sua capacidade de estimular o crescimento de bactérias benéficas do intestino, como bifidobactérias e lactobacilos. As aplicações mais importantes dos XOS em termos de procura atual e potencial do mercado correspondem a ingredientes para alimentos funcionais (por exemplo, em combinação com leite de soja, refrigerantes, chá ou bebidas de cacau, preparações nutritivas, laticínios, leite em pó e iogurtes, doces, bolos, bolachas, doces, pudins, geleias e preparações especiais para alimentos saudáveis para idosos e crianças) ou como componentes ativos de preparações simbióticas (Moure et

al., 2006). Entretanto, os XOS apresentam também uma gama de outras propriedades bioativas que incluem atividade antioxidante, propriedades antialérgicas, antimicrobianas, anti-infecciosas, anti-inflamatórias, atividade citotóxica e uma variedade de outras propriedades (Moure et al., 2006) que merecem ser exploradas.

Em 2018, o mercado global de polissacarídeos e oligossacarídeos ultrapassou o valor de 12,2 mil milhões de dólares e espera-se que cresça a uma taxa de crescimento anual composta (CAGR) de 4,8% durante o período entre 2017 e 2026. O uso crescente de prebióticos na indústria de laticínios e suplementos alimentares é o principal motor desse crescimento do mercado. O aumento da conscientização e preocupação com a saúde entre os consumidores tem conduzido a várias mudanças no estilo de vida, incluindo o foco em produtos alimentares saudáveis e nutritivos (www.factmr.com/).

2.1.3. Lenhina

A lenhina é um polímero tridimensional complexo que ocorre predominantemente no xilema da maioria das plantas terrestres, formando cerca de 1/3 da biomassa lenhosa terrestre. Como componente principal da parede celular de traqueídeos, vasos e fibras, a lenhina contribui para a resistência à compressão de caules lenhosos e para a impermeabilização de elementos condutores dentro do xilema (Donaldson, 2001). A lenhina mantém as células da madeira juntas fornecendo extraordinárias características de resistência (Demirbas, 2004). É principalmente hidrofóbica e sua absorção de água é baixa (Pereira et al., 2003). Em geral, o teor de lenhina mostra uma grande variação entre as espécies: nas monocotiledôneas (por exemplo, gramíneas), varia entre 5 e 12%, nas resinosas entre 25 e 35% e nas folhosas entre 15 e 30% (Lourenço & Pereira, 2018).

Os três percussores fenólicos monoméricos principais que dão origem à lenhina são: álcool *p*-cumarílico, álcool coniferílico e álcool sinapílico. Estes monómeros fenilpropanólicos diferem apenas no grau de substituição metoxilo no anel fenólico: álcool *p*-cumarílico (não metoxilado), álcool coniferílico (um metoxilo na posição carbono 3) e álcool sinapílico (dois grupos metoxilo nos carbonos 3 e 5) (Fig. 5). As unidades monoméricas da lenhina são unidas por ligações carbono-carbono (C-C) e carbono-oxigénio (C-O-C) (Sannigrahi et al., 2010; Área & Popa, 2014). Por não ser formada por unidades monoméricas que se repetem, pode ser mais adequadamente descrita como uma macromolécula do que como um polímero (Área & Popa, 2014). Os anéis aromáticos destes álcoois cumarílico, coniferílico e sinapílico são designados,

respectivamente, por *p*-hidroxifenilo (H), guaiacilo (G) e siringilo (S), no qual se baseia a designação dos diferentes tipos de lenhinas (Pereira et al., 2003).

As lenhinas das resinosas são predominantemente do tipo G com quantidades menores de unidades H, as lenhinas das folhosas contêm unidades G e S, e as gramíneas, por outro lado, contêm unidades estruturais de G. S e H (Berlin & Balakshin, 2014; Lourenço & Pereira, 2018). Assim, a relação S/G tem sido o parâmetro de composição de lenhina mais avaliado devido a sua importância nas reações para produção de pasta; é considerado um fator de qualidade de madeira para pasta e um parâmetro de seleção em programas de melhoramento de espécies vegetais (Lourenço & Pereira, 2018).

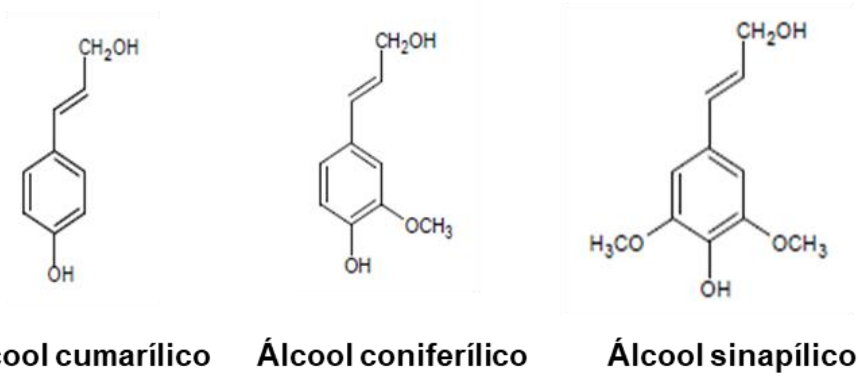

Fig. 5 Estrutura química dos precursores da lenhina

Apesar das características da lenhina como um produto natural com um alto grau de funcionalidades químicas e biofísicas, a sua utilização está ainda subexplorada, sendo as aplicações comerciais usualmente associadas à imagem de um material residual de baixa qualidade (Gosselink et al., 2010). Além de factores económicos, as dificuldades da valorização da lenhina passam também por problemas técnicos relacionados com as suas características estruturais e, em certa medida, pelos próprios métodos de deslenhificação (Vishtal & Kraslawski, 2011).

As lenhinas geradas como subprodutos e que geralmente são usadas como fonte de energia para operar unidades industriais (e.g., fábricas de pasta e papel e biorrefinarias de lenhocelulose) são chamadas de lenhinas técnicas ou lenhinas industriais. As lenhinas técnicas originadas de processos industriais de pasta e papel ("Kraft" ou sulfito) são consideradas principalmente produtos residuais cujas propriedades químicas não são controladas e estão disponíveis em grandes volumes, principalmente em licores Kraft e, em menor extensão, nos licores "ao sulfito". No entanto, existe um grande grupo de lenhinas técnicas obtidas a partir de vários processos de pré-tratamento

da biomassa (existentes industrialmente ou emergentes) (Quadro 2) (Berlin & Balakshin, 2014).

Quadro 2. Características das lenhinas industriais (Berlin & Balakshin, 2014).

Tipo de lenhina	**Escala**	**Química**	**Conteúdo de Enxofre**	**Pureza**
Kraft	Industrial	Alcalina	Moderado	Moderada
Soda	Industrial	Alcalina	Livre	Moderada-Baixa
Lenhosulfonato	Industrial	Ácida	Alto	Baixa
Organosolv	Piloto/Demonstração	Ácida	Livre	Alta
Hidrólise	Industrial/Piloto	Ácida	Baixo-Livre	Moderada-Baixa
SE	Demonstração/Piloto	Ácida	Baixo-Livre	Moderada-Baixa
AFEX	Piloto	Alcalina	Livre	Moderada-Baixa

SE: lenhina obtida após explosão com vapor;
AFEX: lenhina obtida do processo de explosão com amónia

Além da utilização energética da lenhina de baixo valor, existe uma grande diversidade de aplicações industriais de alto valor (Figura 6) (Gosselink, 2011), incluindo usos como novos materiais, matéria-prima polimérica, oligomérica e monomérica (Berlin & Balakshin, 2014). São hoje realidades comerciais, por exemplo, o uso da lenhina ou de seus derivados em aditivos para alimentação animal, agricultura, construção, têxtil, ligantes, dispersantes, e compósitos. Contudo, outras aplicações como a produção de precursores de fibra de carbono, a ampla incorporação da lenhina em misturas poliméricas sintéticas ou a produção de BTX mantém-se como oportunidades a médio prazo com grande valor e potencial de mercado (Berlin & Balakshin, 2014).

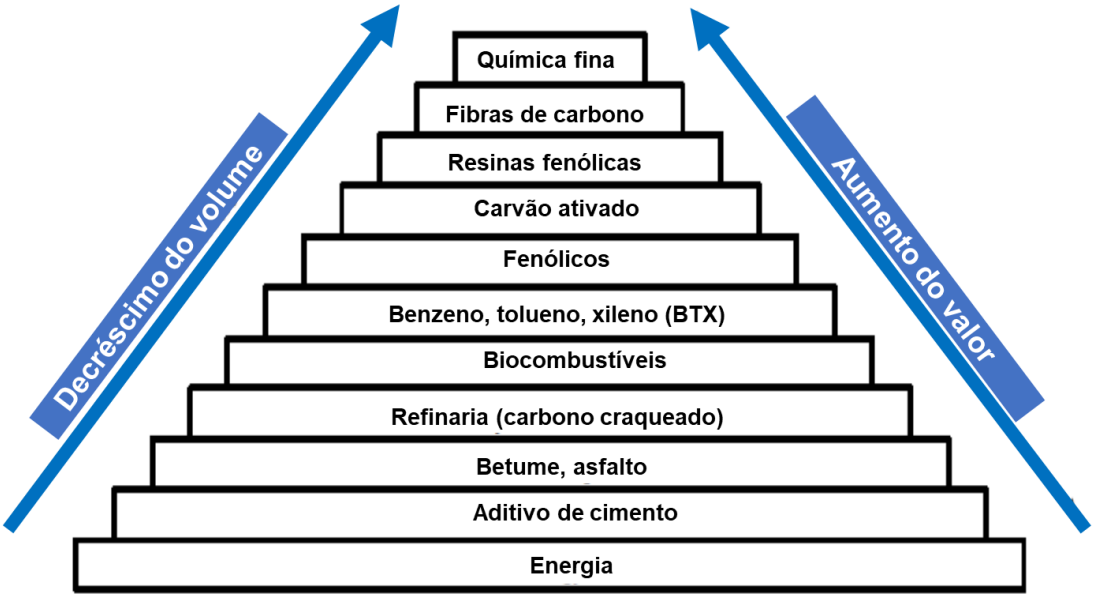

Fig. 6 Potencial aplicações da lenhina (Adaptado de Gosselink, 2011)

2.1.4. Extrativos

Os extrativos compreendem uma grande variedade de compostos químicos, em geral, de baixa massa molecular (Pereira et al., 2003) e podem ser provenientes de duas fontes gerais: 1) metabolitos primários que incluem açúcares, aminoácidos, gorduras simples, vários ácidos carboxílicos entre outros; e metabolitos secundários que são geralmente compostos mais complexos, tais como o amido, sitosterol, terpenóides simples, clorofila, fenilpropanóides, fenólicos; e 2) artefactos oriundos de modificações posteriores dos metabolitos ou mesmo de fontes externas, como, por exemplo, metabolitos de microrganismos ou de líquens (Rowe & Conner, 1979).

Embora, os extrativos representem uma pequena proporção de madeira (≤ 10%) com exceção de madeiras tropicais, onde esse valor pode ser maior (Pereira et al., 2003), eles contribuem de forma significativa para as características das espécies. Em geral, as diferentes classes de extrativos têm comportamentos químicos diferentes (Peng et al., 2010). Os extrativos podem proteger a madeira da decomposição, dar cor e odor à madeira e melhorar as propriedades de resistência. Pelo contrário, podem contribuir para a corrosão de metais em contato com a madeira, inibir a fixação de cimento, colas e acabamentos, ou causar vários problemas durante o fabrico de papel (Rowe & Conner, 1979).

Os extrativos são componentes não-estruturais que estão contidos nas células vegetais, sem estarem ligados quimicamente à parede celular. São compostos orgânicos

solúveis que podem, por exemplo, ser removidos do material lenhocelulósico pelo uso de solventes com polaridade adequada, sem que ocorram mudanças nas características estruturais da célula (Rowe & Conner, 1979; Pereira et al., 2003). Este facto é importante do ponto de vista tecnológico, uma vez que a separação de compostos hidrofílicos e lipofílicos não-estruturais dos materiais lenhocelulósicos pode deixar os sólidos extratados adequados para fracionamento dos compostos estruturais (Gullón et al., 2011; Alves-Ferreira et al., 2019).

Os procedimentos usuais para isolar os extrativos incluem técnicas tradicionais (maceração, decocção, digestão, infusão, ebulição sob refluxo, Soxhlet) e uma ampla gama de técnicas modernas que vem sendo introduzidas nas últimas décadas (extração assistida por micro-ondas, extração assistida por ultra-som, extração com fluido supercrítico, extração líquida pressurizada, extração assistida por enzimas, entre outras) (Brusotti et al., 2014). A obtenção de fitoquímicos na sua forma mais pura possível e em quantidades suficientes, envolve, para além da etapa de extração, várias outras etapas, como triagem farmacológica; identificação, isolamento e caracterização dos compostos bioativos, seguido de sua elucidação estrutural, avaliação toxicológica e avaliação clínica (Sasidharan, et al., 2011). No entanto, essas técnicas são geralmente trabalhosas e caras, havendo, assim, uma necessidade urgente de desenvolvimento de novas técnicas avançadas para a identificação, extração e isolamento de compostos bioativos de plantas (Patra et al., 2018).

As diversidades funcionais apresentadas por estes compostos oferecem inúmeras oportunidades para o desenvolvimento de novos medicamentos e também representam uma excelente fonte de moléculas para a produção de aditivos alimentares, alimentos funcionais, produtos nutricionais e nutracéuticos para as indústrias farmacêuticas e para um número crescente de empresas de alimentos naturais (Patra et al., 2018). O mercado de extratos vegetais está segmentado de acordo com as suas possíveis aplicações e incluem principalmente especiarias, óleos essenciais, sabores e fragrâncias, fitomedicamentos e fitoquímicos. A América do Norte, seguida pela Ásia-Pacífico têm dominado o mercado global de extratos de plantas que, por sua vez, tem sido impulsionado pela crescente mudança no comportamento do consumidor que, cada vez mais, tem se interessado por produtos naturais com benefícios para a saúde (transparencymarketresearch.com).

Compostos fenólicos

Os compostos fenólicos são considerados um dos mais importantes grupos dos metabolitos secundários das plantas, devido a sua grande participação no desenvolvimento de processos morfológicos, fisiológicos e de reprodução (Działo et al., 2016). Os compostos fenólicos compreendem um ou mais anéis aromáticos com grupos hidroxila nas suas estruturas (Minatel et al., 2017). Em geral, são agrupados em várias categorias como ácidos fenólicos e análogos, flavonóides, taninos, estilbenos, lignanas entre outros (Fig 7) (Fresco et al., 2006).

Os ácidos fenólicos são geralmente divididos em dois grupos principais: ácidos benzóicos, contendo sete átomos de carbono (C6-C1) e ácidos cinâmicos compreendendo nove átomos de carbono (C6-C3). Os estilbenos são compostos fenólicos que exibem dois anéis aromáticos ligados por uma ponte de eteno. Os taninos são compostos polifenólicos geralmente classificados em duas classes: os taninos hidrolisáveis e os não hidrolisáveis (ou condensados). Os taninos hidrolisados são polifenóis complexos que podem ser degradados em açúcares e ácidos fenólicos por meio de alterações de pH ou hidrólise enzimática ou não enzimática. As unidades básicas de taninos hidrolisáveis do tipo poliéster são o ácido gálico e seus derivados. As lignanas são dímeros (com duas unidades C6-C3) resultantes da ligação cauda-cauda de duas unidades de álcool coniferílico ou sinapílico (Fresco et al., 2006). A estrutura química dos compostos flavonóides é composta por dois anéis aromáticos ligados por uma ponte de três carbonos (C6-C3-C6). e suas principais subclasses são as flavonas, flavonóis, flavan-3-óis, isoflavonas, flavanonas e antocianidinas. No estado fisiológico, os flavonóides ocorrem geralmente em associação com o açúcar como glicosídeos (Del Rio et al., 2013).

As propriedades bioativas dos compostos fenólicos também têm sido associadas à prevenção de algumas doenças crónicas e degenerativas como doenças cardiovasculares, diabetes tipo II ou distúrbios neurodegenerativos como doença de Alzheimer e Parkinson (Santos-Buelga et al., 2019). Estes compostos também têm ganhado cada vez mais reconhecimento pelas suas propriedades quimiopreventivas e quimioterápicas, pois contribuem para a indução de apoptose ao impedir o ciclo celular e regulação do metabolismo de agentes carcinógenos (Huang et al., 2009). Entretanto, são necessários mais estudos mecanísticos, bem como um conhecimento preciso das concentrações dos agentes quimiopreventivos e seus metabolitos para entender completamente como essas moléculas interagem com os processos fisiológicos e patológicos humanos (Fresco et al., 2006; Huang et al., 2009; Del Rio et al., 2013).

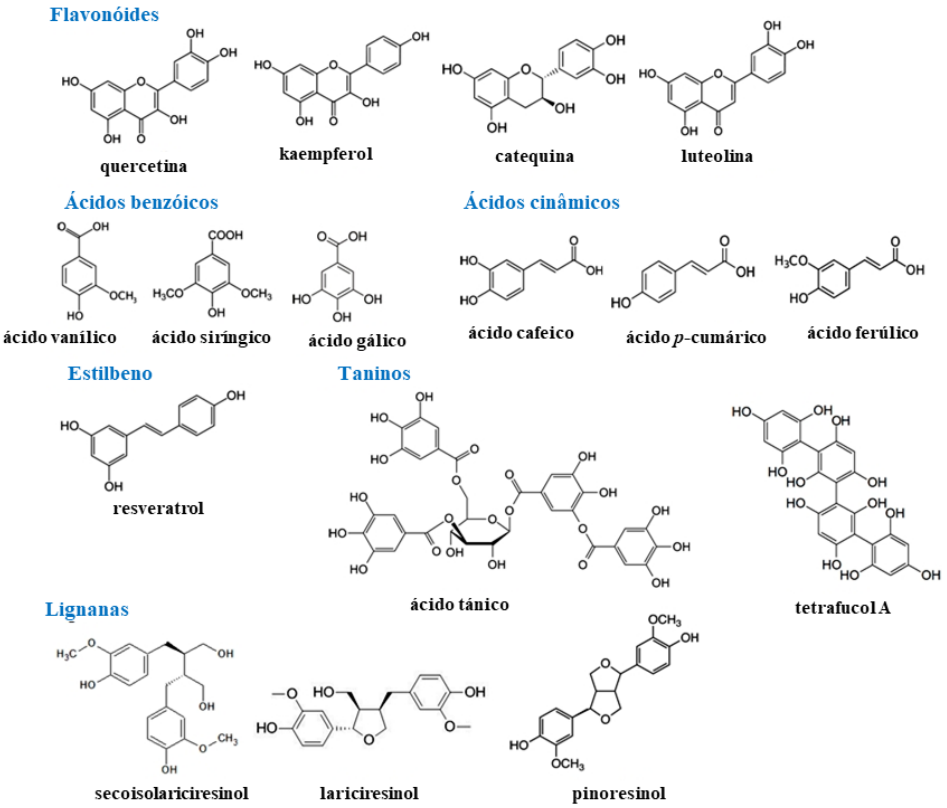

Fig. 7 Estruturas moleculares dos compostos fenólicos mais comuns (Adaptado de Działo et al., 2016)

3. Cistus ladanifer: biomassa modelo para o uso em biorrefinaria

Cistus ladanifer L. (Fig. 8), comumente conhecida em Portugal como esteva e na literatura em inglês como rockrose, é uma espécie arbustiva da família Cistaceae que encontra-se distribuída numa ampla gama de gradientes latitudinais, altitudinais e climáticos especialmente no Sudoeste Europeu (Portugal e Espanha) (Fig. 9). Ocorre em solos ácidos (preferencialmente derivados de substratos silicosos), pouco desenvolvidos e com deficiência nutricional (Rossine-Oliva et al., 2016); cresce preferencialmente em zonas secas e de grande insolação onde pode originar grandes e densas populações, vulgarmente conhecidas por estevais (Biodiversidade Terrestre, 2019).

Fig. 8 Planta de *Cistus ladanifer*

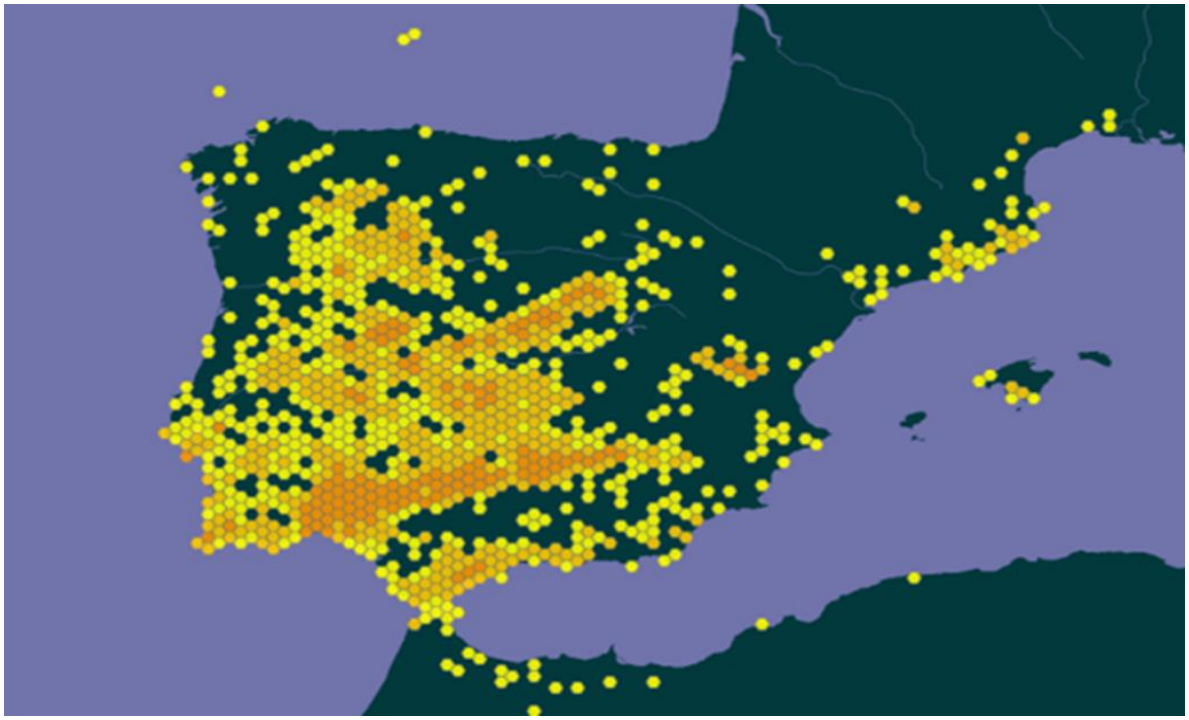

Fig. 9 Distribuição de *C. ladanifer* no Sudoeste Europeu (*Cistus ladanifer* L. GBIF Secretariat, 2017) (https://www.gbif.org/species/6437976)

As suas folhas enquanto jovens encontram-se impregnadas pelo lábdano, uma resina pegajosa excretada especialmente no verão (Biodiversidade Terrestre, 2019).

Entretanto, a presença desta resina também confere à esteva uma alta capacidade inflamável (Delgado et al., 2001). Dessa forma, por ser uma espécie abundante em áreas não cultivadas ou exploradas como pastagens (Castro & Freitas, 2009) poderá causar problemas no verão, sobretudo no que respeita ao risco de incêndios (Nunes et al., 2005). Por outro lado, *C. ladanifer* tem um papel importante como ponte na recuperação de povoamentos florestais após o fogo, fornecendo inóculo micorrízico disponível para colonizar as raízes das árvores à medida que o novo povoamento é desenvolvido (Hernández-Rodríguez et al., 2013).

Assim, o ecossistema de *C. ladanifer* também fornece alta produção de espécies de cogumelos comestíveis, alguns deles em grande procura devido ao seu interesse gastronómico (Hernández-Rodríguez et al., 2015). Além disso, a esteva também é considerada promissora para programas de fitorremediação e revegetação de solo contaminado em climas semiáridos, pois é capaz de colonizar estes solos com grande capacidade de tolerância e adaptabilidade às condições climáticas adversas (secas, altas temperaturas) (Rossine-Oliva et al., 2016; Santos et al., 2016).

Além dessas capacidades adaptativas, *C. ladanifer* é também particularmente interessante para a extração de óleos essenciais (Marioti et al., 1997; Gomes et al., 2005; Teixeira et al., 2007). Os óleos essenciais da esteva são muito apreciados pelo seu odor balsâmico e poder fixador. Dos produtos sintetizados pelas plantas, os óleos essenciais estão entre os mais valiosos, encontrando grande aplicação comercial. Destaca-se também o facto desta planta ser potencialmente rica em compostos bioativos (Chaves et al., 1998; Andrade et al., 2009) com interesse nas indústrias farmacêutica e fitoquímica.

Embora seja uma matéria-prima versátil, a esteva é também um exemplo de espécie de cultura subutilizada e atualmente, não possui qualquer atividade de exploração regular, a não ser como fonte de óleos essenciais e lábdano para a indústria da perfumaria ou para fins ornamentais. Assim, faz sentido investigar novas aplicações para valorizar este recurso natural subestimado, cujas potencialidades biológicas e adaptativas são enormes (Raimundo et al., 2018).

Por exemplo, a valorização da biomassa remanescente da extração (resíduos ou subprodutos) permitiria melhorar a rentabilidade económica das empresas que processam a esteva para a indústria da cosmética e perfumaria, uma vez que estas são habitualmente de pequena dimensão e muitas vezes o seu verdadeiro potencial e eficiência não estão explorados. Tendo em conta que os processos utilizados para a extração de óleos essenciais são relativamente suaves, i.e., trata-se de processos aquosos a temperaturas moderadas, os resíduos/subprodutos obtidos podem ser utilizados para a produção de uma gama alargada de coprodutos. Isso constituiria uma

mais-valia importante para estas unidades, usualmente focadas num só produto, dando-lhes a possibilidade de produção de novos produtos.

4. Processos de fracionamento da biomassa lenhocelulósica

Embora os pré-tratamentos da biomassa tenham sido inicialmente desenvolvidos para facilitar a hidrólise da celulose, atualmente eles tendem a ser entendidos como processos de fraccionamento para a valorização integral da biomassa (Carvalheiro et al., 2008; Gírio et al., 2010), dado que é necessário valorizar todas as frações da biomassa para que se obtenha uma valorização industrial sustentável.

As propriedades estruturais e a composição da biomassa lenhocelulósica, tais como cristalinidade da celulose, grau de polimerização, teor de lenhina e grau de acetilação das hemiceluloses são afetadas por diferentes pré-tratamentos em graus diferentes (Biswas & Ahring, 2016). O pré-tratamento é necessário para alterar o tamanho e a estrutura macroscópica e microscópica da biomassa, bem como a sua composição e estrutura química submicroscópica, de modo que os tratamentos subsequentes sejam facilitados, por exemplo, a hidrólise da fração de polissacarídeos para açúcares monoméricos possa ser alcançada mais rapidamente e com maiores rendimentos (Mosier et al., 2005). Assim, cada estratégia de pré-tratamento tem diferentes efeitos sobre a estrutura lenhocelulósica e, de uma maneira geral, podem operar sob várias condições para maximizar a recuperação seletiva do produto, enquanto minimizam a formação de inibidores para os processos subsequentes de hidrólise e fermentação (Agbor et al., 2011; Xu & Sun 2016).

Os métodos de pré-tratamento/fraccionamento incluem processos físicos (moagem e separação granulométrica e densimétrica), químicos (ácidos, alcalinos, solventes orgânicos e líquidos iónicos), físico-químicos (auto-hidrólise e explosão a vapor), biológicos ou a combinação dessas abordagens (Ajao et al., 2018). Os Quadros 3 e 4 apresentam um resumo das vantagens e desvantagens de alguns dos principais métodos de pré-tratamento de materiais lenhocelulósicos.

O pré-tratamento deve atender a alguns requisitos, tais como: alta recuperação de açúcares, directamente após hidrólise enzimática, produção zero ou em quantidades muito limitadas de produtos de degradação, baixa ou nenhuma utilização de químicos, ser rentável para aplicações industriais e aplicável a uma ampla variedade de biomassas, independentemente da sua composição química (Sun & Cheng, 2002; Biswas & Ahring, 2016). Embora alguns métodos de pré-tratamento/fraccionamento já se encontrem bem

estabelecidos com progressos ao nível de otimizações e desenvolvimento de processos alternativos, de facto, há ainda limitações que precisam ser resolvidas (Carvalheiro et al, 2013); mais precisamente em relação às perdas de material devido à degradação, ao uso de produtos químicos e consequente necessidade de recuperação e reciclagem destes produtos, a problemas de corrosão, exigindo equipamentos e manutenção dispendiosos, e também às questões ambientais associadas ao uso de produtos químicos (Biswas & Ahring, 2016).

Quadro 3. Vantagens e desvantagens dos principais métodos de fracionamento da biomassa lenhocelulósica (Adaptado de Biswas et al., 2011; inclui também opinião da autora)

Pre-tratamentos	Vantagens	Desvantagens	Referências[a]
Auto-hidrólise	Uso da água como solvente, não requer nenhum produto químico ou recuperação; utiliza temperaturas ligeiramente mais baixas do que a explosão com vapor; pequena formação de inibidores	Os produtos solubilizados podem estar em concentrações baixas; baixa capacidade de remoção da lenhina; pode envolver grandes volumes de água	1-4
Explosão com vapor	Uso de vapor; em geral não requer catalisadores químicos, não necessita de reciclagem; os requisitos energéticos são relativamente baixos	Destruturação incompleta da lenhina e polissacarídeos; solubilização parcial das hemiceluloses; pode produzir quantidades apreciáveis de inibidores em temperaturas altas	2, 3, 5, 6
Hidrólise ácida diluída	Solubiliza pricincipalmente as hemiceluloses. Pode solubilizar parcialmente a celulose e, altera a estrutura da lenhina; aumenta a área superficial	Uso de ácidos torna o processo mais dispendioso (custo do ácido e problemas de corrosão); mais perigoso; requer produtos alcalinos para neutralização; forma inibidores em concentrações mais altas; equipamentos e custos operacionais altos	2-4, 7, 8
Líquidos iónicos	Dissolução muito eficaz da celulose; as perdas de hidratos de carbono são geralmente baixas e os produtos de degradação são significativos apenas em condições severas.	Alto custo de solvente; custo da regeneração de solventes,	9, 10
Alcalino	Aumenta a área superfícial; altera a estrutura da lenhina levando à sua dissolução melhora a hidrólise enzimática; possibilidade de operar a temperaturas mais baixas	lenhinas de baixa qualidade como subproduto; custos adicionais devido à necessidade de ajuste de pH e lavagem exaustiva dos sólidos	11, 12
Organosolv	Acelera a deslenhificação por dissociação dos iões [H^+]; lenhina e celulose reativas de alta qualidade e uma corrente aquosa contendo as hemiceluloses e açúcares derivados	Necessidade de reciclagem do solvente para economia do processo; nalguns casos o solvente residual pode causar inibição microbiana; perigos de explosão, preocupações ambientais, de saúde e de segurança	2, 8
Biológicos	Usa condições suaves e baixa energia; aplicação ambientalmente sustentável	Taxa de hidrólise lenta, podendo levar várias semanas/ meses; ineficiente para aplicação industrial;	2

[a] 1 - Hendriks & Zeeman, 2009; 2- Agbor et al., 2011; 3 – Alvira et al., 2010; 4 – Brodeur et al., 2011; 5 – Gnansounou & Dauriat, 2010; 6 – Hamelinck et al., 2005; 7 – Mosier et al., 2005; 8 – Kumar et al., 2009; 9 – Kim et al., 2009; 10 – Rodriguez et al., 2011; 11 – Chandra et al., 2012; 12 – Cardona & Sánchez, 2007.

Quadro 4. Efeitos de diferentes processos de pré-tratamento nas composições e estruturas de materiais lenhocelulósicos (Adaptado de. Sun et al., 2016; inclui também opinião da autora)

Pré-tratamentos	Remoção da lenhina	Remoção das hemiceluloses	Aumento da área superficial	Descristalinização da celulose	Aumento de porosidade	Formação de produtos de degradação
Auto-hidrólise	B	A	M	B	M	B
Explosão com vapor	B	A	A	B	A	M/A
Ácido	M	A	A	-	M	A
Líquidos iónicos	M	B	A	A	A	B
Alcalino	A	A	A	-	A	B
Organosolv	A	B/M	A	-	M	B
Biológicos[1]	A	M	A	-	A	B

A : Alta ; M : Média ; B : Baixa; [1] os processos biológicos têm tempos de reacção muito mais elevados do que os processos químicos ou físico-químicos

4.1. Tratamentos hidrotérmicos

Os processos hidrotérmicos, em especial a auto-hidrólise (liquid hot water, LHW) e a explosão com vapor, baseiam-se na utilização de água ou vapor, ou ambos, e calor para o tratamento da biomassa (Carvalheiro et al, 2013). A geração de protões resultantes da auto-ionização da água e do ácido acético atuam como catalisadores para a hidrólise das hemiceluloses, causando a hidrólise dos grupos acetilo que são libertados como ácido acético. Deste modo, ocorre a dissolução total ou parcial das hemiceluloses e a sua conversão em oligossacarídeos e monossacarídeos (Vallejos et al, 2017).

Após o pré-tratamento para hidrólise das hemiceluloses, além do licor hemicelulósico, é também obtido um resíduo sólido rico em celulose e lenhina que poderão ser ainda separados ou usados em conjunto (Fig 10).

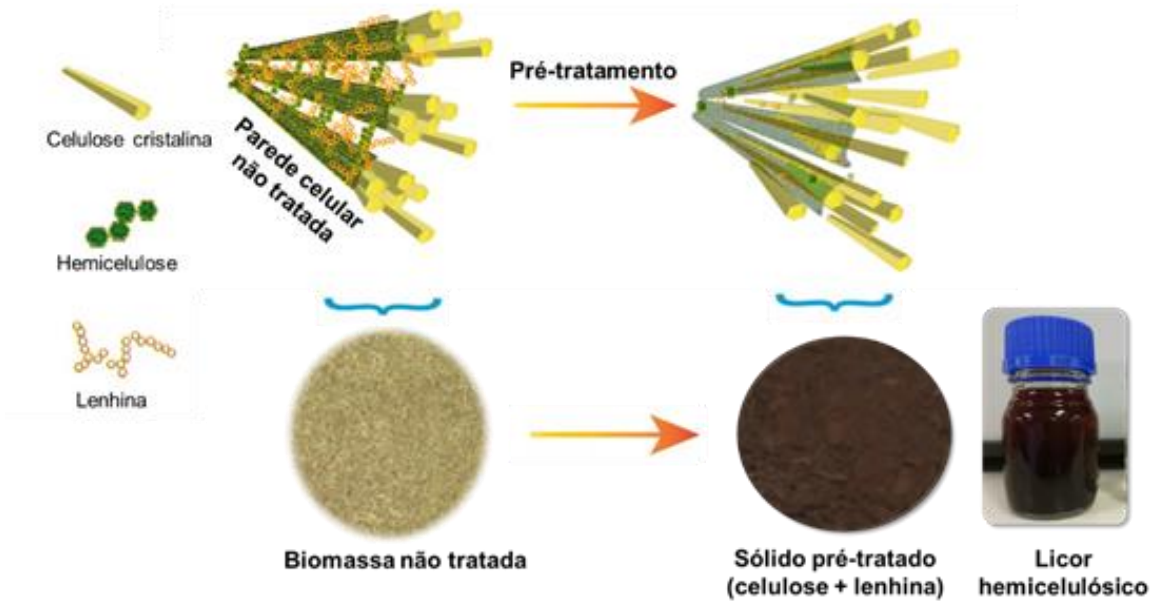

Fig. 10 Efeito do pré-tratamento na estrutura da parede celular da biomassa lenhocelulósica, bem como no aspecto. Este tipo de tratamento conduz à solubilização das hemiceluloses (fração líquida), enquanto a celulose e lenhina não degradada permanecem na fração sólida (Modificado de Biswas et al., 2015)

4.1.1. Auto-hidrólise

A auto-hidrólise utiliza água em temperaturas e pressões elevadas para manter a sua forma líquida, a fim de promover a desintegração e separação da matriz lenhocelulósica (Brodeur et al., 2011). Dessa forma ocorre uma hidrólise relativamente

elevada das hemiceluloses e uma baixa formação de inibidores, enquanto a celulose e lenhina não são praticamente degradadas (Carvalheiro et al., 2004). A libertação dos grupos acetilo conforme referido anteriormente juntamente com o hidrogeniões resultantes da auto-ionização da água, conduzem à acidificação da fase líquida ajudando a catalisar a despolimerização das hemiceluloses produzindo assim oligossacarídeos e açúcares monoméricos que, subsequentemente, são parcialmente degradados a aldeídos (Mosier et al., 2005). Desse modo, os produtos da auto-hidrólise são uma mistura de oligossacarídeos, monossacarideos, ácidos acéticos, derivados do furano, (principalmente furfural e hidroximetilfurfural (HMF) e alguns compostos fenólicos resultantes da despolimerização parcial da lenhina (Biswas e Ahring, 2016) e extrativos. A fase sólida é constituída por hemiceluloses residuais, lenhina e celulose (Chiaramonti et al., 2012; Carvalheiro et al., 2013; Alves-Ferreira et al., 2019).

As reações de hidrólise das hemiceluloses ocorrem a temperaturas tipicamente na gama de 150–230 °C (Chiaramonti et al., 2012). Abaixo de 150 °C, a celulose e a lenhina não são modificadas substancialmente, mas pode ainda ocorrer uma conversão apreciável das hemiceluloses em oligossacarídeos (Biswas & Ahring, 2016). Altas temperaturas e maior tempo de residência permitem uma maior remoção de massa e, embora as temperaturas elevadas (> 200 °C) possam permitir uma hidrólise quase total das hemiceluloses, a quantidade de açúcares presentes nos licores resultantes da extração pode diminuir rapidamente devido ao aumento da formação de produtos de degradação (Liu et al., 2012b).

A razão líquido/sólido (LSR) também é uma variável importante a ser considerada em tratamentos hidrotérmicos embora seja pouco estudada e o seu efeito sobre a dissolução das hemiceluloses possa também ser ainda controverso. Em geral, a proporção entre líquido e sólido pode variar entre 2:1 e 100:1 (p/p), dependendo da matéria-prima, sendo que os valores mais usuais estão em torno de 10:1 (Vallejos et al., 2017). Na auto-hidrólise, uma LSR alta pode favorecer a extração de hemiceluloses porque aumenta a difusão de reagentes (água e ácido acético gerado) dentro do material lenhocelulósico. No entanto, isso pode diminuir a viabilidade económica do processo em grande escala, não só aos custos de operação (OPEX), dado o elevado consumo de água, que levam à obtenção de hidrolisados mais diluídos mas, essencialmente pelos maiores gastos energéticos para o aquecimento da mistura reacional e para a recuperação das hemiceluloses (Vallejos et al., 2017). Mais, LSR elevados levam também a maiores custos de investimento (CAPEX) que tornam o processo também menos atrativo em termos económicos. Por outro lado, uma LSR baixa resulta no uso mais eficiente da água e consequentemente numa redução dos custos operacionais

(Vallejos et al., 2017) e de CAPEX, para além de que conduz a um aumento da concentração dos grupos acetilo, podendo tornar a despolimerização mais eficaz.

O factor de severidade R_0 é um parâmetro empírico usado para comparar os resultados de pré-tratamentos aquosos realizados em diferentes condições de temperatura e tempo (Overend e Chornet, 1987). Assim, é possível combinar num único parâmetro o efeito dessas variáveis operacionais na extensão da conversão ou rendimento em produto (Carvalheiro, 2005).

O factor de severidade para processos hidrotérmicos denominado ordenada de reação (R_0) em geral é expresso usando a seguinte função Log:

$$R_0 = \int_0^t exp(\frac{T(t) - 100}{14.75})dt$$

Nesta expressão, T é a temperatura da reação, t é o tempo de residência (min), 100 é a temperatura abaixo da qual os processos hidrolíticos são pouco significativos e 14,75 é um parâmetro empírico que expressa a influência média da temperatura na reação, ou seja,
por cada aumento de 10 ºC da temperatura, e mantendo todas as outras variáveis constantes, a velocidade da reação duplica.

Os extrativos e compostos inorgânicos são removidos com baixas severidades, enquanto a desestruturação das fibras a nível fibrilar ocorre quando a severidade aumenta. A despolimerização e a solubilização de hemiceluloses começam com valores de severidade inferiores a 2,5, ao passo que valores até 3,8 causam uma rápida diminuição no grau de polimerização da celulose (Vallejos et al., 2017). Os XOS são os principais produtos presentes nos hidrolisados provenientes da auto-hidrólise de biomassas ricas em xilanas sob condições moderadas (Gullón et al., 2010). Os tratamentos de maior severidade conduzem a produtos com um menor grau de polimerização, mas também aumenta a sua hidrólise em xilose (Moure et al., 2006) bem como a degradação desta última.

4.1.2. Explosão com vapor

Na explosão com vapor, a biomassa é tratada com vapor de água saturado a uma pressão elevada em geral, aplicada por alguns minutos. Após o tempo de reação definido, a pressão é reduzida rapidamente, provocando a descompressão súbita e consequente explosão do material. Em geral, neste tipo de pré-tratamento são aplicadas

temperaturas até 240 °C e as principais alterações físicas e estruturais para além da remoção química das hemiceluloses permitem aumentar significativamente a digestibilidade enzimática da biomassa (Mosier et al., 2005; Hendriks & Zeeman, 2009). No entanto, entre as desvantagens apresentadas por esse tratamento estão a maior degradação das hemiceluloses, por exemplo comparativamente à auto-hidrólise, levando à formação de produtos de degradação, que dependem das condições do tratamento e da matéria-prima (Chiaramonti et al., 2012). A explosão com vapor pode ser realizada em descontínuo ou em sistemas contínuos. Os reatores para funcionamento descontínuo são versáteis, e de controle simples, sendo particularmente usados em escala laboratorial com quantidades relativamente pequenas de biomassa (0,1 - 1 kg). Por outro lado, pese embora não seja possível operar este processo em continuo, dado o tempo de operação curto (ordem dos segundo), é possível trabalhar com taxas de alimentação muito altas que tornam o processo quase contínuo, com apreciáveis vantagens às escalas piloto e de demonstração (Chiaramonti et al., 2012).

4.2. Hidrólise ácida

O principal objetivo dos pré-tratamentos ácidos é solubilizar o fração hemicelulósica da biomassa e tornando assim também a celulose mais acessível à acção de enzimas. Este tipo de pré-tratamento pode ser realizado com ácido concentrado ou diluído para romper a estrutura rígida do material lenhocelulósico (Alvira et al., 2010; Bordeur et al., 2011). O ácido concentrado pode permitir obter alto rendimento de açúcares com temperatura baixas, hidrolisando assim as hemiceluloses e celulose (Rabemanolontsoa & Saka, 2016). Embora o uso de ácidos concentrados seja muito eficaz na hidrólise da biomassa lenhocelulósica, este tipo de pré-tratamento requer materiais de construção dos reatores caros, além de ser um processo potencialmente perigoso (Chiaramonti, 2012). Além disso, a recuperação do ácido é fundamental para a economia do processo. A hidrólise ácida diluída apresenta a vantagem de menor consumo de ácido, porém são necessárias temperaturas mais elevadas para a hidrólise das hemiceluloses e mais altas ainda para obter uma hidrólise apreciável da celulose resultando também nestes casos numa degradação extensiva das hemiceluloses (Rabemanolontsoa & Saka, 2016). Assim, a hidrólise ácida diluída aparece como um método mais favorável que o ácido concentrado para aplicações industriais.. Pode ser realizada a altas temperaturas (por exemplo, 180 °C) durante um curto período de tempo; ou a temperaturas mais baixas (por exemplo, 120 °C) para um tempo de retenção mais longo (30 a 90 min) (Alvira et al., 2010). O ácido sufúrico (H_2SO_4) tem sido o mais

comumente usado para pré-tratamento de uma ampla variedade de biomassa, pelo seu baixo custo e elevada eficiência, melhorando significativamente a hidrólise da celulose (Bordeur et al., 2011).

A hidrólise ácida também é um dos processos que tem gerado lenhinas industriais com abundância em unidades industriais de hidrólise ácida de resíduos da madeira e agrícolas no Leste da Europa desde meados da década de 30. A principal via de degradação de lenhina, sob condições ácidas, ocorre por meio da hidrólise das ligações β-O-4. Embora a degradação da lenhina ocorra por meio de intermediários do éter vinílico, estes não se acumulam na lenhina por serem estruturas muito instáveis em meio ácido. Além disso, as reações de condensação e a precipitação dos componentes da lenhina solubilizada sob condições ácidas são mais significativas do que aquelas que ocorrem em processos alcalinos, diminuindo assim a digestibilidade da biomassa pré-tratada (Hendriks & Zeeman, 2009; Berlin & Balakshin, 2014).

A hidrólise ácida requer que os ácidos sejam posteriormente reciclados/neutralizados, produzindo grandes quantidades de resíduos e conduzindo também a perdas dos hidrolisados. De facto, a recuperação do ácido é necessária para alcançar uma melhor economia do processo, embora seja em si um passo consumidor de energia. Outra desvantagem da hidrólise ácida é a formação de diferentes tipos de inibidores, tais como ácidos carboxílicos, furanos e compostos fenólicos, principalmente quando se utiliza ácido concentrado que, por esse motivo, acaba por não ser muito atraente para a produção de etanol, po exemplo (Hendriks & Zeeman, 2009; Alvira et al., 2010; Chiaramonti et al., 2012).

4.3. Métodos alcalinos

A lenhina constitui uma fonte muito importante de compostos com atividades funcionais e com potencial de aplicação em várias indústrias. Entre os processos químicos utilizados para remoção da lenhina (deslenhificação) destacam-se os processos alcalinos que embora afetem principalmente a lenhina também podem afetar as hemiceluloses. Os processos alcalinos apesar de poderem ser realizados a temperaturas baixas, têm a desvantagem de originar lenhinas de baixa qualidade. No entanto, são adequados para aumentar eficientemente a acessibilidade de enzimas à celulose (Chiaramonti et al., 2012).

O mecanismo da hidrólise alcalina consiste na saponificação das ligações ésteres intermoleculares que ligam a lenhina e a xilana, ou seja, os iões hidróxido provocam o inchamento da celulose, a hidrólise da ligação éster e a ruptura das ligações

de hidrogénio intermoleculares entre celulose e hemiceluloses, colocando assim uma parte das hemiceluloses em solução (Sun & Cheng, 2002; Farhat et al., 2017). A degradação da lenhina em condições alcalinas ocorre através da clivagem de ligações β-O-4 que resulta na formação de porções do tipo álcool coniferílico como produto de reação primária, que embora não estejam acumulados na lenhina, sofrem reações adicionais de rearranjo secundário formando vários ácidos (aril) alifáticos. Já os tipos de ligações β-5 e β-1 não podem ser clivadas durante o processamento, mas são transformadas em estruturas do tipo estilbeno que, por sua vez, são estáveis e acumulam-se nas lenhinas alcalinas (Berlin & Balakshin, 2014).

A pré-tramento alcalino também altera o grau de polimerização dos componentes do material lenhocelulósico, provocando mudanças nas propriedades físicas dos sólidos tratados, nomeadamente na área superficial, porosidade e cristalinidade da biomassa (Kim et al., 2016). A extração alcalina é a base do processo de produção de celulose, que está bem estabelecido e processa anualmente 100 milhões de toneladas de madeira (Farhat et al., 2017).

Os agentes alcalinos fortes, tais como o hidróxido de sódio, têm a tendência de dissolver as hemiceluloses a partir da parede celular, embora o núcleo da celulose cristalina também possa ser diminuído através da descristalização. Refira-se também que os agentes alcalinos mais fracos, como a amónia e o hidróxido de cálcio, só libertam hemiceluloses de baixo peso molecular, uma vez que têm menos capacidade para clivar as ligações de hidrogénio entre as hemiceluloses e celulose, bem como as ligações éster entre as hemiceluloses e lenhina. (Xu & Sun, 2016).

As condições para tratamento alcalinos são geralmente menos severas do que outros pré-tratamentos. Pode até ser realizado à temperatura ambiente , mas com o emprego de um maior tempo de residência (Brodeur et al., 2011). Ao contrário dos pré-tratamentos catalisados por ácido, uma parte dos agentes alcalinos são convertidos em sais irrecuperáveis ou que são incorporados na biomassa pelas reações de pré-tratamento (Mosier et al., 2005).

O pré-tratamento com hidróxido de sódio é um dos métodos alcalinos mais aplicados e vem sendo estudado desde 1900. Esta base forte é evidenciada pela sua eficácia na solubilização das hemiceluloses e lenhina e por alcançar uma maior eficiência de hidrólise enzimática quando comparada a outros pré-tratamentos alcalinos (Kim et al., 2013). A combinação do tratamento alcalino com outros métodos, como oxidação húmida, explosão com vapor ou explosão com amónia também tem sido reportada em vários estudos (Brodeur et al., 2011).

Um dos exemplos de processos alcalinos mais conhecidos é o processo Kraft, que utiliza hidróxido de sódio e sulfureto de sódio e é tradicionalmente utilizado na

produção de pastas para papel (Sridach, 2010; Gosselink, 2011). Este é dos processos que produz maior volume de lenhina devido a alta taxa de deslenhificação, mas é pouco específico e muito poluente (Sridach, 2010). A cor escura da pasta celulósica é uma outra desvantagem desse processo. No entanto, o desenvolvimento de técnicas de branqueamento nos anos 1940 permitiu a consolidação dessa tecnologia (Rodrigues et al., 2018). Após a deslenhificação, as fibras de celulose (pasta) são lavadas, branqueadas e secas. O licor negro, composto por hemiceluloses, lenhina e grande parte dos elementos inorgânicos usados na digestão, é concentrado por evaporadores multi-efeito e queimado na caldeira de recuperação para produzir vapor (Ajao et al., 2018).

A implementação de biorrefinarias integradas nas fábricas de pasta Kraft tem sido um dos tipos de biorrefinaria integrada à indústria de base florestal mais estudadas. Essa integração também é um meio para que as fábricas de pasta e papel diversifiquem e aumentem as suas receitas à queda da procura de papel, da intensa concorrência global e dos altos preços da energia (Ajao et al., 2018).

4.4. Organosolv

Os processos organosolv baseiam-se na utilização de solventes orgânicos como agentes de deslenhificação sendo uma alternativa interessante às tecnologias comerciais atuais, uma vez que conduzem a uma fase sólida enriquecida em celulose e a licores contendo produtos da dissolução da lenhina e da hidrólise das hemiceluloses livres de enxofre (Xu et al., 2006). A lenhina pode ser isolada como produto puro e não como subproduto ao final do ciclo de produção do biocombustível (Nitsos et al., 2018). Essas misturas aquosas com solventes orgânicos podem ainda ser utilizadas em combinação com outros catalisadores, geralmente, pode obter-se um alto rendimento de xilose com a adição de ácido (Su & Cheng, 2002; Bozell et al., 2011). Os diferentes solventes orgânicos, bem como o pH de reação utilizados nos diversos métodos organosolvs podem produzir lenhinas muito diferentes em suas propriedades químicas e bioquímicas (Berlin & Balakshin, 2014).

Para reduzir o custo de pré-tratamento, os solventes devem ter uma boa solubilidade para a lenhina e serem fáceis de recuperar. Os álcoois de cadeia curta e ácidos orgânicos, como etanol, ácido fórmico e ácido acético, parecem ser os solventes mais promissores para o pré-tratamento de biomassa lenhocelulósica (Chen et al., 2015). Contudo, o glicerol, um solvente orgânico com elevado ponto de ebulição e subproduto da indústria de oleoquímicos e de biodiesel, tem também tido bastante interesse (Sun et al., 2015).

Os solventes utilizados no processo precisam ser drenados do reator, evaporados, condensados e reciclados (Sun & Cheng, 2002; Chiaramonti et al., 2012). A remoção de solventes do sistema é também necessária porque o solvente pode inibir o crescimento de microrganismos nos processos de fermentação (Sun & Cheng, 2002). Além, disso, a economia global destes processos depende da reciclagem dos solventes. O etanol e a acetona, por exemplo, apresentam a vantagem de serem facilmente recicláveis por destilação e serem eles próprios produtos das biorrefinarias (Carvalheiro et al., 2008).

Os processos organosolv têm a vantagem de produzir lenhinas com baixa percentagem de contaminantes (menor teor em cinza e em açúcares) e, em geral, com menor peso molecular e maior hidrofobicidade, e que podem ainda ser utilizadas posteriormente para obtenção de produtos de valor acrescentado (Holladay et al., 2007; Rodrigues et al., 2018). Alguns estudos também têm demonstrado que esses processos mantém a estrutura do núcleo da lenhina praticamente inalterada (El Hage et al., 2009). Por outro lado, este processo também apresenta algumas desvantagens como a ocorrência das reações hidrolíticas a pressões mais elevadas e a necessidade de lavagem dos sólidos pré-tratados com solvente orgânico, para evitar a reprecipitação da lenhina dissolvida (Novo et al., 2011).

A tecnologia organosolv mais estudada, Alcell, foi implantada à escala industrial no leste do Canadá durante os anos 80. Esse processo pode ser realizado com etanol aquoso em acidez moderada, uma vez que nenhum ácido exógeno é adicionado e a acidez do meio resulta da formação de ácidos orgânicos durante o processo. A lenhina Alcell é praticamente isenta de enxofre e tem uma baixa quantidade de impurezas como açúcares e cinzas em comparação com lenhinas Kraft (Berlin & Balakshin, 2014). Com o declínio da Alcell, a instalação piloto foi comprada posteriormente pela Lignol Innovations Corp., uma empresa dedicada à produção de produtos de lenhina de alto valor, coprodutos de xilose e bioetanol (Mabee et al., 2006) e que entretanto foi vendida à empresa Fibria (Grupo Suzano, Brasil).

De facto, a maior parte dos trabalhos com organosolv concentra-se no uso de um ácido mineral (geralmente sulfúrico) como catalisador. Assim, o processo Lignol está entre os mais importantes nesta categoria (Nitsos et al., 2018). A abordagem Lignol tem sido utilizada para separar lenhina, componentes de hemiceluloses e extrativos da fração celulósica da biomassa lenhosa. A fração celulósica resultante é altamente suscetível à hidrólise enzimática, gerando rendimentos muito altos de glicose. As condições preferidas dependem da natureza da matéria-prima a ser processada, mas geralmente estão entre 180 e 195 °C, com tempo de residência de 30 a 90 min, numa concentração de etanol de 35 a 70% (p/p). O pH do licor pode variar de pH 3,8 a pH 2,0. Um menor pH do licor, uma

temperatura mais alta e um tempo de residência mais longo geralmente reduzirão a quantidade de xilose que pode ser recuperada (Arato et al., 2005). Uma outra abordagem intermediária entre a catálise ácida com ácidos fortes e a reação não catalisada é a adição de ácidos orgânicos fracos como ácido fórmico e ácido acético à mistura etanol/água (Hideno et al., 2013; Agnihotri et al., 2015; Nitsos et al., 2018).

De um modo geral, o sucesso do processo organosolv é determinado pela qualidade e quantidade dos produtos e também pelo sistema de recuperação para os solventes utilizados (Rodrigues et al., 2018). Entretanto essa tecnologia exige altos investimentos de capital e tem tido dificuldade de se atingir a escala industrial (Smolarski, 2012; Rodrigues et al., 2018).

4.5. Hidrólise enzimática

As celulases e as xilanases englobam uma classe de enzimas cuja função primária é hidrolisar as ligações glicosídicas dos principais polissacarídeos estruturais da planta: celulose e xilanas. Ao converter celulose e xilana em açúcares constituintes, essas enzimas desempenham um papel essencial no sistema digestivo de herbívoros e na reciclagem de carbono fixado fotossinteticamente (Gilbert & Azlewood, 1993). No entanto, as enzimas para aplicações industrial deverão apresentar uma elevada estabilidade em condições extremas de temperatura, pH e salinidade. Alguns microrganismos (bactérias e fungos) vivem nesses ambientes e são considerados fontes de enzimas com potenciais aplicações biotecnológicas. As bactérias, por exemplo, podem degradar os substratos celulolíticos de diferentes modos. As anaeróbias, degradam a celulose usando celulossomas, enquanto as bactérias aeróbias produzem enzimas extracelulares que difundem-se livremente para alcançar o substrato (Quiroz-Castañeda & Folch-Mallol, 2013).

As xilanas são hidrolisadas por uma variedade de enzimas hidrolíticas e, pelo facto da sua estrutura ser mais complexa do que a da celulose, exige um grande número de enzimas diferentes para induzir a hidrólise eficiente. Como as hemiceluloses exigem estruturas relativamente amorfas, tornam-se mais acessíveis às enzimas hidrolíticas. Consequentemente, a atividade específica das xilanases é de 2 a 3 ordens de grandeza superior que a hidrólise da celulose cristalina pelas celulases (Gilbert & Azlewood, 1993). Além da interação de várias enzimas hidrolíticas para a hidrólise das xilanas, a ação das enzimas acessórias são importantes para a remoção dos grupos laterais terminais do polímero ou oligómero. Assim, a hidrólise das xilanas é desempenhadas pelas seguintes enzimas (Carvalheiro, 2005):

a) "Endo-xilanases: hidrolisam principalmente as ligações internas de xilose β-1,4 da cadeia de xilano principal;
b) Exo-xilanases: hidrolisam as ligações de xilose β-1,4, produzindo xilobiose;
c) β-xilosidases: produzem xilose a partir de xilobiose e de XOS de cadeia curta;
d) α-L-arabinofuranosidases: hidrolisam as extremidades não-redutoras de arabinofuranose;
e) α-glucuronidases hidrolisam os resíduos de ácido glucurónico e os seus ésteres 4-*O*-metilo;
f) Acetilxilano-esterases: hidrolisam as ligações acetil-éster;
g) Feruluílo-esterases: hidrolisam as ligações éster entre os substituintes de arabinose e o ácido ferúlico;
h) p-cumaroílo-esterases hidrolisam as ligações éster entre os substituintes de arabinose e o ácido *p*-cumárico".

Por outro lado, a degradação e sacarificação eficientes da celulose requerem uma reação sinergéica de três classes de enzimas celulolíticas (Abdel-Rahman et al., 2011; Quiroz-Castañeda & Folch-Mallol, 2013):

a) Endo-β-1,4-glucanases (EG; EC 3.2.1.3): hidrolisam aleatoriamente as ligações β-1,4-glicosídicas intramoleculares acessíveis das cadeias de celulose e inserem uma molécula de água na ligação β-(1,4), criando um novo par de extremidades de corrente redutoras e não redutoras;

(b) Exo-β-1,4-glucanases ou celobiohidrolases (CBH; EC 3.2.1.91): catalisam a hidrólise sucessiva de resíduos das extremidades redutoras e não redutoras da celulose, liberando moléculas de celobiose como produto principal, que são hidrolisadas pelas β-glicosidases a glucose. Elas representam 40 a 70% do total componente do sistema celulase, e são capazes de hidrolisar a celulose cristalina.

(c) β-Glucosidases (β-G; EC 3.2.1.21) (celobioses): hidrolisam celobiose a glucose, a fim de eliminar a inibição da celobiose.

O contacto eficiente das celulases com a celulose são factores importantes para o sucesso da hidrólise enzimática, para além das condições de reação, como pH e temperatura. A eficiência da hidrólise enzimática depende do tipo de mistura enzimática utilizada, da atividade enzimática e da concentração de enzimas (Biswas & Ahring, 2016). A conversão satisfatória de celulose usando uma menor quantidade de enzimas, deve estar acima dos 75% (Biswas & Ahring, 2016).

A hidrólise enzimática de materiais lenhocelulósicos é influenciada não apenas pela eficiência das enzimas, mas também pelas características físicas, químicas e morfológicas da matéria-prima (Sun et al., 2016). Entre os factores limitantes da hidrólise enzimática podemos citar grau de polimerização da celulose, a cristalinidade da celulose, a área de superficial disponível do substrato, teor e distribuição da lenhina, teor de hemiceluloses, tamanho das partículas, porosidade e espessura da parede celular (Alvira et al., 2010). Em síntese, a taxa inicial de hidrólise enzimática da celulose está estreitamente associada ao seu índice de cristalinidade, uma vez que a celulose com baixa cristalinidade apresenta uma sacarificação enzimática muito maior do que as amostras de celulose com alta cristalinidade. O tamanho de partícula e o volume de poros ou porosidade, mostram grande influência na área superficial específica acessível dos materiais lenhocelulósicos (Sun et al., 2016). Um aumento da porosidade pelo processo de pré-tratamento pode melhorar significativamente a hidrólise (Alvira et al., 2010), pois poros suficientemente grandes podem acomodar tanto grandes como pequenos componentes enzimáticos, mantendo assim a ação sinergética do sistema enzimático da celulase (Sun et al., 2016). A presença de lenhina e hemiceluloses também reduz a eficiência da hidrólise, embora, a recuperação de açúcares hemicelulósicos nos sólidos pré-tratados seja interessante para obter maior produção total de açúcares fermentescíveis a partir do sólidos (Sun et al., 2016).

A hidrólise enzimática, quando realizada em cargas com alto teor de sólidos (≥15% de sólidos, m/m), oferece vantagens comparada às conversões realizadas com cargas baixas ou moderadas de sólidos, o que inclui o aumento das concentrações de açúcar e produto final e redução dos custos operacionais e de capital (Modenbach & Nokes, 2013). Contudo, esse tipo de abordagem apresenta também algumas limitações, nomeadamente a disponibilidade de água, dificuldades com a mistura, transferência de calor e aumento da concentração de inibidores. Dessa forma, é necessária a utilização de reatores adequados para trabalhar com misturas que apresentam uma elevada consistência, bem como a utilização de enzimas robustas capazes de manterem sua atividade na presença de possíveis inibidores ou mesmo o desenvolvimento de pré-tratamentos que não produzam inibidores (Modenbach & Nokes, 2013).

Atualmente, a sacarificação enzimática utilizando celulases é a abordagem mais eficaz, viável, e adequada do ponto de vista ecológico para a hidrólise da biomassa, em que a produção razoável de açúcares a partir de matérias-primas celulósicas atinge mais de 90% em ambientes hidrolíticos otimizados (Chandel et al., 2018).

5. Desenvolvimento e produção do ácido láctico

5.1. Ácido láctico

O ácido láctico (LA) (ácido 2-hidroxipropanóico) é um ácido orgânico amplamente distribuído na natureza, constituído por um átomo de carbono quiral e que existe em duas formas enantioméricas (D e L) (Fig. 11). Este composto foi descoberto em 1780 no leite azedo por C.W. Scheele e em 1881 foi obtido por Fermi por fermentação, resultando na sua produção industrial (Martinez et al., 2013). O comportamento químico do ácido láctico é determinado pelas suas propriedades físico-químicas, como o caráter ácido em meio aquoso, a reatividade funcional associada à presença dos grupos carboxilo e hidroxilo, o que lhe confere grande versatilidade de reação, e a atividade óptica assimétrica do carbono 2 (Gao et al., 2011; Martinez et al., 2013).

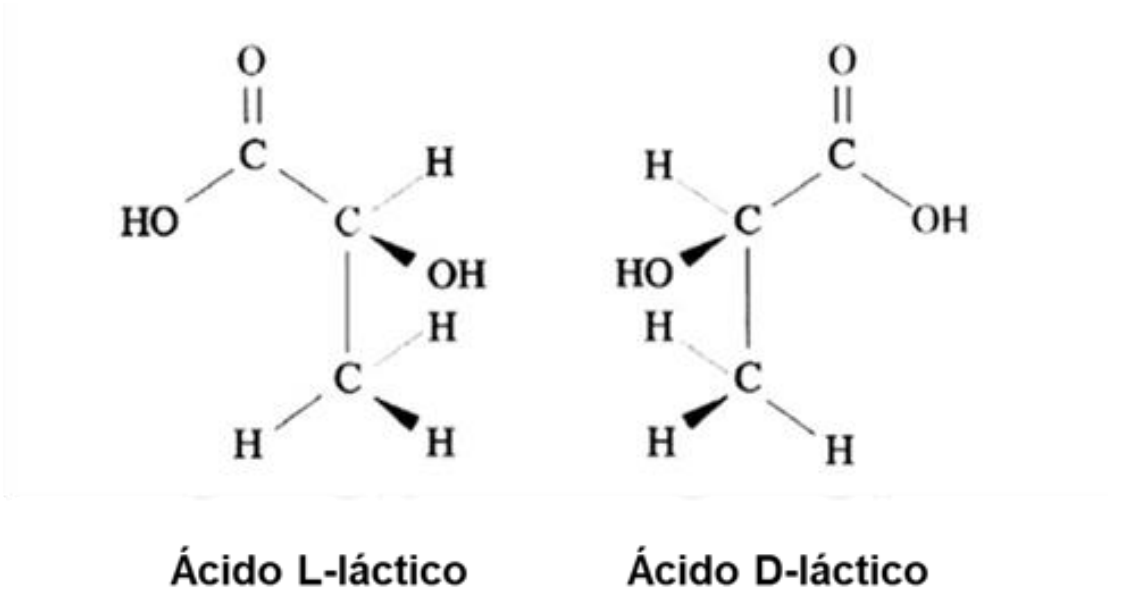

Fig. 11 Estrutura dos isómeros L(+) e D(-) do ácido láctico

A existência do LA nestas duas formas torna a utilização de um deles ou da mistura racêmica um aspecto importante. Os isómeros puros são mais valiosos do que a forma racêmica, uma vez que cada um tem a sua aplicação industrial específica. Por exemplo, as indústrias alimentares e farmacêuticas têm uma preferência pela forma L, o único que pode ser metabolizado pelo corpo humano. Por outro lado, a indústria química requer um dos isómeros puros ou uma mistura de ambos, de acordo com a aplicação (Åkerberg et al., 1998; Abdel-Rahman et al., 2011; Martinez et al., 2013).

A procura de ácido láctico tem aumentado consideravelmente devido às aplicações promissoras do ácido poliláctico (PLA), um polímero semi-cristalino

biodegradável e termoestável, empregado como uma alternativa sustentável aos plásticos obtidos a partir de petroquímicos (Abdel-Rahman et al., 2011; Reddy et al. 2008). A pureza óptica do ácido láctico a partir dos seus isómeros é crucial para as propriedades físicas do PLA (Okano et al., 2010) que existe sob três formas: ácido poli L-láctico (PLLA), ácido poli- D-láctico (PDLA) e ácido poli DL-láctico (PDLLA). Embora o PLLA seja adequado para o uso industrial, a sua aplicação é limitada devido a sua baixa temperatura de fusão (175 °C) (Tashiro et al., 2011; Liu et al., 2018). Entretanto, a mistura entre os homopolímeros PLLA e PDLA permite a cocristalização em um novo arranjo, formando um estereocomplexo com um ponto de fusão marcadamente mais alto (230 °C) e com um melhor desempenho mecânico (Ikada et al. 1987; Henton et al., 2005). Esta descoberta aumentou consideravelmente a produção de ácido D-láctico (Tashiro et al., 2011; Liu et al., 2018), tendo em conta que este além de se mostrar interessante para a produção de PLA, também apresenta um papel fundamental na melhoria da qualidade e desempenho de poli (L-lactídeo) convencional, uma vez que possui uma alta resistência ao calor (Mai et al., 2018).

No cenário atual da química verde, da preocupação com o meio ambiente e da bioeconomia, o PLA tem se tornado muito atraente para o mercado, dada a sua degradação rápida no meio ambiente em subprodutos de toxicidade muito baixa, sendo eventualmente convertidos em dióxido de carbono e água. Cabe ressaltar, que pode ser o polímero com a mais ampla gama de aplicações devido à sua capacidade de ser cristalizado sob tensão e termicamente, modificado por impacto, preenchido, copolimerizado e processado na maioria dos equipamentos de processamento de polímeros. Além disso, o PLA também possui excelentes características organolépticas e é excelente para contacto com alimentos, nomeadamente para embalagens (Henton et al., 2005).

O ácido láctico pode ser usado numa variedade muito grande de produtos (Fig. 12), podendo ser obtido por via química ou bioquímica. A síntese química do ácido láctico baseia-se principalmente na hidrólise de lactonitrilo por ácidos fortes, produzindo, entretanto, apenas a mistura racémica (John et al., 2007). Contudo, devido às preocupações ambientais e a natureza limitada do setor petroquímico, os processos biotecnológicos para a produção de produtos derivados de ácido láctico é o método preferencial (Gao et al, 2011). Além disso, a produção biotecnológica de ácido láctico oferece diversas vantagens em relação à síntese química, nomeadamente o baixo custo de substratos, baixa temperatura de produção e baixo consumo de energia (John et al., 2007) e pode constituir-se como uma dos principais produtos de grande volume “bulk” das biorrefinarias a par ou invés dos biocombustiveis.

As principais etapas de produção biotecnológica do LA incluem, geralmente, a sua fermentação, remoção da biomassa microbiana e proteína, recuperação e purificação de ácido láctico, concentração do produto e remoção de cor (Hentont et al., 2005; Wee et al., 2006). Para obtê-lo biotecnologicamente de maneira competitiva por via bioquímica, é ainda necessário resolver questões ao nível de microrganismos produtores com alto desempenho, bem como da redução dos processos de fermentação (Wee et al., 2006).

A economia da produção de LA pela via fermentativa depende de muitos factores, nomeadamente o custo das matérias-primas que pode ser significativo. Assim, têm sido vários os materiais considerados como substratos interessantes e renováveis, incluindo subprodutos de indústrias agrícolas e indústrias alimentares, biomassa rica em amido, soro de leite, glicerol, biomassa de algas e em particular a biomassa lenhocelulósica (Abdel-Rahman et al. , 2011; 2013). A biomassa lenhocelulósica é uma matéria-prima promissora para a produção de LA, considerando a sua grande disponibilidade, sustentabilidade e baixo custo potencial em comparação com os açúcares refinados.

Fig. 12 Potenciais usos do ácido láctico nas diferentes indústrias (Adaptado de Wee et al., 2006)

Neste âmbito, a conversão bioquímica da biomassa lenhocelulósica requer várias etapas de processamento a fim de converter polissacarídeos em açúcares monoméricos. Estes açúcares podem ser fermentados em ácido láctico por estirpes com vários graus de eficiência. Desse modo, o processo convencional para produção de ácido láctico a partir de biomassa lenhocelulósica apresenta quatro passos principais (Abdel-Rahman et al., 2011):

a) Pré-tratamento - destruturação da estrutura lenhocelulósica;
b) Hidrólise enzimática - despolimerização da celulose/hemiceluloses em açúcares fermentescíveis (como glucose e xilose), por meio de enzimas hidrolíticas;
c) Fermentação - metabolização dos açúcares em ácido láctico, por microorganismos;
d) Separação e purificação de ácido láctico – a purificação é primordial para atender aos padrões das aplicações comerciais.

Uma das desvantagens da fermentação láctica é a obtenção de uma solução relativamente diluída de ácido láctico devido à inibição dos microorganismo pelo próprio ácido produzido. Além disso, o meio de fermentação contém sempre muitas impurezas, incluindo a biomassa celular, outros ácidos orgânicos e nutrientes não consumidos. Nesse sentido, a recuperação e purificação do LA a partir de meios fermentativos requer várias etapas e operações unitárias, o que consequentemente contribui para o aumento do custo de produção (Kanungnit & Rattanaphanee, 2011). Existem várias abordagens para a recuperação e purificação do LA a partir dos meios fermentativos entre as quais se destacam: destilação, extração líquida, esterificação, processos de sal insolúvel, os processos com membranas, eletrodiálise, osmose inversa, nanofiltração, métodos térmicos e abordagens utilizando resinas de troca iónica (Henton et al., 2005; Kanungnit & Rattanaphanee, 2011; Ghaffar et al., 2014).

5.2. Fermentação

A gama de produtos que são produzidos por fermentação ao nível industrial está a expandir-se para além dos tradicionais compostos de baixo-volume e alto valor, como os produtos farmacêuticos, e começa a competir com a produção sintética tradicional de produtos químicos. À medida que a fermentação permite a obtenção de produtos químicos de maior volume e de baixo valor, torna-se necessário maximizar a

eficiência e minimizar os custos e os resíduos para competir eficazmente com as opções tradicionais (John et al., 2007).

O processo bioquímico de produção de LA utiliza microrganismos e/ou reações enzimáticas para converter um substrato fermentescível em produtos recuperáveis. A fermentação é realizada, mais usualmente, em solução aquosa, com os produtos finais presentes em concentrações moderadas. As hexoses, particularmente a glucose, são os substratos de fermentação mais frequentemente utilizados, ainda que as pentoses, glicerol também possam ser utilizados desde que os microorganismo tenham capacidade de os converter (Kamm & Kamm, 2006). Nos processos fermentativos, as soluções de açúcar são suplementadas com nutrientes e inoculadas com o microorganismo selecionado. Assim, é necessário selecionar as condições de fermentação mais favoráveis, em termos de temperatura, pH, arejamento, agitação, etc., de acordo com o microorganismo utilizado (Martinez et al., 2013). No entanto, no caso da utilização de hidrolisados obtidos a partir de materiais lenhocelulósicos, a presença dos inibidores, também interfere na eficiência da fermentação, sendo necessária a avaliação da necessidade de destoxificação (Palmqvist & Hahn-Hägerdal, 2000a).

O processo de fermentação, utilizando biomassa sólida pré-tratada e hidrolisados, pode ser conduzido separadamente, após a sacarificação enzimática do sólido (*SHF-Separate Hydrolysis and Fermentation*). Contudo, durante a hidrólise enzimática, a atividade da celulase pode ser inibida pelos produtos formados, i.e., pela celobiose e, em menor grau, pela glucose. Por essa razão, foram desenvolvidos vários métodos para reduzir essa inibição, incluindo o uso de concentrações altas de enzimas, a suplementação com β-glucosidases durante a hidrólise e a remoção de açúcares durante a hidrólise por ultrafiltração ou, em especial recorrendo-se à estratégia de sacarificação e fermentação simultâneas (*SSF-Simultaneous Saccharification and Fermentation*) (Sun & Cheng, 2002).

O processo SSF permite a hidrólise e fermentação dos açúcares numa única etapa, ou seja, os açúcares redutores liberados pela enzima são simultaneamente convertidos no produto final pelo microorganismo, o que reduz fortemente a inibição pelo produto (Iyer & Lee, 1999; Sun & Cheng, 2002; John et al., 2009; Martinez et al., 2013). Assim, comparando os processos SHF e SSF, o último apresenta as seguintes vantagens: (1) aumento da taxa de hidrólise pela conversão de açúcares que inibem a atividade da celulase; (2) necessidade de menor carga de enzima; (3) maior rendimento do produto; (4) menores requisitos para condições de esterilidade, uma vez que a glucose é removida imediatamente e o subproduto é produzido; (5) menor tempo de processo; e (6) o uso de um único reator. Contudo, algumas limitações

precisam de ser consideradas: (1) as temperatura de hidrólise e fermentação podem não ser compatíveis; e (2) inibição das enzimas pelo produto formado (Iyer & Lee, 1999; Sun & Cheng, 2002; Martinez et al., 2013).

No que se refere aos aspectos limitantes do processo de SSF, para atenuá-los exige-se o emprego de microrganismos robustos e preferencialmente termotolerantes, uma vez que a temperatura ótima para a sacarificação enzimática é mais elevada que a temperatura ótima para o crescimento dos microrganismos em geral. De facto, muitas das bactérias produtoras de ácido láctico são termotolerantes, possibilitando, assim, que a temperatura de operação do SSF possa ser levada para condições próximas do ótimo da enzima celulase, tornando o processo global mais eficiente, especialmente no uso de enzimas (Iyer & Lee, 1999; John et al., 2009).

A recuperação preliminar do produto de fermentação deve exigir etapas como filtração, destilação ou extração. As etapas finais de recuperação e purificação do produto devem ser exclusivas de cada produto (Kamm & Kamm, 2004)

5.3. Microrganismos produtores de ácido láctico

O ácido láctico pode ser produzido por diferentes microrganismos, entre os quais bactérias, fungos, leveduras, cianobactérias e algas (Abdel-Rahman et al., 2013). Contudo, é de salientar que cerca de 90% da literatura sobre a produção de LA está focada na fermentação bacteriana (John et al., 2007). As bactérias produtoras de LA incluem as do tipo selvagem e as geneticamente modificadas que, por sua vez, podem ser divididas em quatro produtores principais: bactérias lácticas (LAB), estirpes de *Bacillus*, *Escherichia coli* e *Corynebacterium glutamicum* (Abdel-Rahman et al., 2013). As LAB podem ser classificadas em espécies homofermentativas e heterofermentativas. As LAB homofermentativas metabolizam hexoses, produzindo lactato como principal produto (> 90%) e as espécies heterofermentativas metabolizam tanto as hexoses como as pentoses conduzindo a diferentes produtos finais (i.e. CO_2, lactato e acetato ou etanol) (Zotta et al., 2018). Tradicionalmente, o LA tem sido produzido usando as LAB; no entanto, a maioria dessas estirpes não é capaz de usar xilose como fonte de carbono e apresentam requisitos da fonte de azoto exigentes e complexos. Além disso, poucas podem converter os açúcares em ácido D-láctico, o isómero necessário para atingir o ácido poliláctico termoestável (Utrilla et al., 2012).

Contudo, estão a ser desenvolvidas e/ou manipuladas com grande sucesso várias estirpes, com potencial de produção de ácido D-láctico opticamente puro. A maioria desses resultados é obtido usando meios de cultura complexos, como Luria-Bertani (LB)

e MRS ou recorrendo-se a agentes de neutralização caros, o que se torna impraticável em termos de fermentações em larga escala (Liu et al., 2014). Nesse sentido, a utilização de estirpes transformadas, como a *E. coli* oferece boas perspectivas para a conversão de recursos renováveis em produtos de valor acrescentado, como é o caso do ácido láctico. As estirpes de *E. coli* de tipo selvagem têm as vias metabólicas para produzir lactato (D(+) ou L(-), sob condições anaeróbicas, e em comparação com as LAB têm a capacidade de utilizar várias fontes de carbono (Martinez et al., 2017).

5.3.1. Microrganismos transformados: a estirpe E. coli JU15

Utrilla et al. (2012) descreveram a utilização de estirpes transformadas derivadas da estirpe selvagem *Escherichia coli* MG1655, com a capacidade de produzir D-lactato praticamente como único produto fermentativo, com elevado rendimento e produtividade. O facto de estas estirpes poderem utilizar pentoses, constitui uma vantagem adicional com grande relevância para as biorrefinarias e para a produção de PLA, já que a maior parte dos microrganismos são incapazes de utilizar pentoses como fonte de carbono e energia (Hahn-H¨agerdal et al., 2007), limitando a possibilidade de utilização completa dos hidratos de carbono da biomassa lenhocelulósica.

A dificuldade de muitos microrganismos crescerem em pentoses, ocorre em função do baixo rendimento de ATP que se obtém com estes açúcares. De um modo geral, os intermediários orgânicos do metabolismo, como o piruvato ou seus derivados, servem como receptores de eletrões para manter o equilíbrio redox geral durante o crescimento anaeróbico das bactérias. Em *E. coli*, a conversão de glucose em piruvato produz 2 ATPs, enquanto o metabolismo de uma xilose, em piruvato, produz apenas 0,67 de ATP, devido à necessidade de um ATP para o transporte da xilose e outro para a fosforilação da xilose. Dessa forma, o rendimento de ATP de 0,67 por pentose é apenas o suficiente para fornecer energia de manutenção, mas não o suficiente para suportar o crescimento de *E. coli* em meio mínimo. Assim, as enzimas piruvato-formato-liase e acetato-cinase são essenciais para o crescimento anaeróbio de *E. coli* em xilose, pois permitem a conversão de piruvato a acetil-coenzima A que, por sua vez, se converte em acetato. A conversão da xilose em acetato gera um ATP adicional, aumentando o rendimento de 0,67 para 1,5 (Hasona et al., 2004).

Todavia, a transformação da *E. coli* em estirpes que produzam maioritariamente ácido láctico, consiste na deleção do gene que codifica a enzima piruvato formato liase (*pflB*) (Zhou et al., 2003; Zhou et al., 2006; Zhu et al., 2007), que traz como consequência a incapacidade do mutante *pflB* crescer em xilose anaerobicamente, pois há um

rendimento insuficiente de ATP, mesmo que haja suplementação com acetato (Hasona et al., 2004).

Neste contexto, Utrilla et al. (2009) obtiveram a partir da bactéria selvagem *E. coli* MG1655 uma estirpe recombinante designada CL3. A deleção dos genes pflB adhE frdA permitiu não só a produção homofermentativa de D-lactato com um rendimento em glucose de 95%, mas também melhorias nas velocidades específicas de crescimento, de consumo de glicose e de produtividade volumétrica (Utrilla et al., 2009). Em virtude das características notáveis apresentadas pela nova estirpe CL3, foram realizadas novas intervenções no seu genoma, a fim de obter eficiência também na conversão da xilose em lactato. Desse modo, eliminou-se o sistema de transporte da xilose dependente de ATP (XylFGH), originando, assim, uma outra estirpe melhorada, JU01 (*E. coli* MG1655 ΔpflB ΔadhE ΔfrdA ΔxylFGH) (Utrilla et al., 2012). JU01, de facto, apresentou um aumento na taxa de crescimento específico, mas não demonstrou diferenças significativas na taxa de consumo de xilose. Assim sendo, a linhagem JU01 foi submetida a um processo de evolução adaptativa, utilizando xilose em meio mineral, para aumentar a pressão seletiva e assim obter mutantes com melhor capacidade de crescerem em xilose.

Após várias transferências em duas concentrações diferentes de xilose (40 e 120 g/L), foi selecionada uma estirpe eficiente para produzir D-lactato a partir deste açúcar. As ferramentas de proteómica quantitativa e sequenciamento do genoma revelaram mudanças importantes na concentração de enzimas nas vias dos ácidos glicolíticos e tricarboxílicos. O sequenciamento genómico completo da estirpe sujeita ao processo de evolução adaptativa identificou uma mutação pontual no gene gatC, que resultou na mudança de serina para leucina na posição 184 da proteína GatC. A eliminação de GatC num número de estirpes e a inserção da mutação no gene da estirpe não evoluída, confirmou a sua atividade como transportador de xilose e demonstrou que essa é a mutação responsável pelo fenótipo de alto consumo de xilose na estirpe evoluída (Utrilla et al., 2012). É de salientar que as estirpes modificadas foram selecionadas de acordo com a sua resistência à canamicina, uma vez que o plasmídeo, pKD4, utilizado para eliminar o gene referente ao sistema de transporte de xilose dependente de ATP, continha uma cassete de canamicina com regiões de homologia aos genes a serem inativados. A incorporação da cassete no genoma bacteriano, substituindo grande parte do gene inativado, permite identicar as células recombinantes, de acordo com a resistência a esse antibiótico. Dessa forma, durante os ensaios de bioconversão utilizando JU15 é possível suplementar o meio de cultura com canamicina para se evitar contaminações por outras estirpes bacterianas.

A nova estirpe mutante, designada JU15 (MG1655, ΔpflB, ΔadhE, ΔfrdA, ΔxylFGH, gatCS184L, ΔmidarpA, Δreg 27.3 kb) apresentou 95% do rendimento teórico máximo e uma produtividade volumétrica em D-LA de 0,53 g/L.h , utilizando meio suplementado com xilose e acetato. A taxa de consumo de xilose de JU15 foi duas vezes maior quando comparada com a de JU01 (Utrilla et al. 2012). JU15 também tem apresentado desempenho relevante em hidrolisados lenhocelulósicos contendo misturas de hexoses e pentoses e com uma adição mínima de nutrientes (Utrilla et al. 2016).

5.4. Compostos inibidores

Durante o pré-tratamento dos materiais lenhocelulósicos, além dos açúcares solúveis (principalmente glucose, xilose e arabinose) também são produzidos compostos potencialmente tóxicos para os microrganismos (Palmqvist & Hahn-Hägerdal, 2000b; Jönsson et al., 2013). Estes compostos inibidores são divididos em três grupos principais com base na sua origem: ácidos fracos, derivados de furano e compostos fenólicos (Palmqvist & Hahn-Hägerdal, 2000b). O ácido acético é formado principalmente pela hidrólise de grupos acetilo das hemiceluloses, enquanto os furanos são tipicamente formados a partir da degradação dos monossacarídeos; o furfural é gerado pela desidratação de pentoses, tais como arabinose e xilose; e o HMF é o componente gerado pela desidratação das hexoses. Uma fração dos furanos também é degradada em ácidos orgânicos (ácidos fórmico e levulínico) (Almeida et al., 2009; Chiaramonti et al., 2012; Jönsson et al., 2013) (Fig. 13).

Nos hidrolisados hemicelulósicos também podem estar presentes outros produtos, como os iões inorgânicos, que podem ser originários da própria biomassa, os produtos químicos adicionados durante o pré-tratamento e os produtos provenientes do condicionamento ou dos equipamentos usados no processo. Contudo, nalgumas situações, a presença de concentrações moderadas destes iões, assim como dos ácidos alifáticos podem ter um efeito positivo. Por exemplo, estão também descritas melhorias na produção de etanol ou da produção de biomassa e de xilitol na presença destes iões (Larsson et al., 1999; Duarte et al., 2005; Jönsson et al., 2013).

Os efeitos individuais dos inibidores para as células microbianas estão relacionados com a sua estrutura e hidrofobicidade. Estas características determinam a capacidade intrínseca destes compostos de penetrar nas membranas celulares e causar perturbações (Monlau et al., 2014). Os compostos fenólicos e furanos são considerados os mais perigosos para a proliferação celular, enquanto os ácidos orgânicos (acético, fórmico e levulínico) são ligeiramente menos tóxicos (Sivagurunathan et al., 2017).

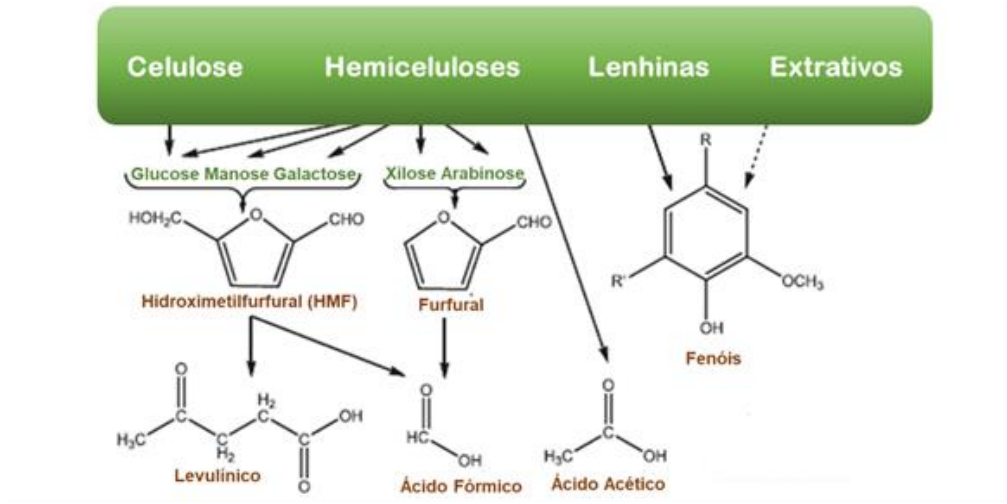

Fig. 13 Esquema indicando as principais vias de formação dos compostos inibidores. Os furanos e os ácidos alifáticos são produtos da degradação dos açúcares, enquantos a lenhina e os extrativos são fontes de compostos fenólicos (Adaptado de Jönsson et al., 2013)

A capacidade do furfural de causar mutações no DNA de microrganismos tem sido há muito reportada (Zdzienicka et al., 1978; Khan & Hadi 1993). Os grupos aldeídos reativos podem provocar uma elevação de espécies reativas de oxigénio, normalmente geradas nas mitocôndrias, causando danos oxidativos às células dos microrganismos (Almeida et al., 2009). Manter a integridade celular é muito importante para que os produtos obtidos em processos fermentativos estejam protegidos e para que se evite gastos da energia da célula em reparações dos danos celulares (Almeida et al., 2007). Os furanos, dependendo da concentração, também têm sido associados a um menor rendimento e produtividade volumétrica em etanol, inibição no crescimento de leveduras ou aumento da fase de latência (Duarte et al., 2005; Almeida et al., 2007; Quémeneur et al., 2012). A inibição da fermentação do etanol pelos furanos tem a ver com a inibição das enzimas álcool-desidrogenase (ADH), piruvato-desidrogenase (PDH) e aldeído-desidrogenase (ALDH) (Modig et al., 2002). Assim, as estratégias para evitar um impacto negativo dos compostos inibidores nos microrganismos, incluem a optimização do pré-tratamento para minimizar a formação desses compostos, a selecção e adaptação das estirpes, bem como uma tecnologia de fermentação apropriada para otimizar a sua conversão *in situ* a produtos menos tóxicos (Almeida et al., 2009).

Relativamente aos ácidos alifáticos, a sua toxicidade está relacionada com a concentração da forma não-dissociada (Duarte et al., 2005). A forma dissociada desses ácidos depende da constante de dissociação (p*K*a) específica para cada um deles. O pKa é o valor de pH em que as concentrações da forma não-dissociada e dissociada dos

ácidos são iguais, tornando a capacidade tampão do ácido mais alta (Palmqvist &Hahn-Hägerdal, 2000a). Caso o pH do meio esteja abaixo da constante de dissociação do ácido em questão, a concentração da sua forma não-dissociada aumenta. Os ácidos fracos não-dissociados são lipossolúveis e por difusão podem penetrar pela membrana plasmática das células microbianas, tornando-se, assim, dissociados no protoplasma que, em geral, apresenta pH neutro. Dessa forma, ocorre uma diminuição do pH intracelular e uma queda na produção de hidrogénio podendo gerar a morte celular (Pampulha & Loureiro-Dias, 1989; Verduyn et al., 1990; Palmqvist & Hahn-Hägerdal, 2000a). Em síntese, a tolerância aos ácidos fracos depende essencialmente do pH do meio de cultura e do arejamento (Carvalheiro, 2005).

Os compostos fenólicos têm sido descritos como capazes de agir sobre as membranas biológicas, causando a perda de integridade e afetando assim a sua capacidade de servir como barreira seletiva (Heipieper et al., 1994). O efeito negativo desses compostos no crescimento de leveduras (Duarte et al., 2005) e a correlação negativa entre o seu peso molecular e a fase de latência e (Quéméneur et al., 2012) também têm sido observados.

5.5. Destoxificação

Os hidrolisados lenhocelulósicos variam no seu grau de inibição, e os diferentes microrganismos apresentam diferentes tolerâncias a inibidores (Palmqvist & Hahn-Hägerdal, 2000b). Assim, os efeitos tóxicos causados pelos diversos inibidores no metabolismo microbiano, dependem da estirpe, do tipo de inibidores e das condições de cultura, e devem-se mais ao efeito combinado dos diversos compostos presentes, do que ao efeito de um único composto individual (Taherzadeh et al., 1997; Zaldivar e Ingram, 1999; Zaldivar et al., 1999; Palmqvist et al., 1999). Dessa forma, é difícil prever/estabelecer gamas de concentrações que afetem o crescimento e/ou produção de metabolitos.

Existem várias abordagens possíveis para minimizar os efeitos dos inibidores, nomeadamente a utilização de condições de hidrólise em que a sua formação seja evitada (por exemplo, processos suaves como a auto-hidrólise), promover a adaptação dos microrganismos aos hidrolisados ou proceder à sua remoção antes da fermentação (destoxificação). Nos casos em que a destoxificação é mesmo necessária, não é possível propor um método universal para a remoção de todo o tipo de compostos em função dos motivos já referidos.

A destoxificação por *overliming* (calagem) é um método bem estabelecido para reduzir a toxicidade dos hidrolisados. Teoricamente, o processo pode remover componentes inibidores por precipitação, converter quimicamente componentes tóxicos em formas não tóxicas e/ou adicionar alguma substância ao hidrolisado (por exemplo, iões de cálcio) que aumente a capacidade de fermentação dos microrganismos (Ranatunga et al., 2000). O *overliming* convencional é, geralmente, realizado pela adição de uma base ($Ca(OH)_2$ ou NaOH) até alcançar um pH alto (normalmente na gama de 9 a 11) e após um tempo pré-estabelecido (30-60 min), bem como temperatura (25 ou 60 °C), o pH é diminuído a um nível adequado para a fermentação (Martinez et al., 2001; Mohagheghi, et al., 2006; Mateo et al., 2013). O processo overliming, a temperaturas elevadas, reduz a quantidade de base necessária para a destoxificação, a quantidade de ácido necessária para o reajuste do pH e minimiza os problemas associados a contaminações (Martinez et al., 2001).

O ajuste de pH, também muitas vezes designado por neutralização, consiste em acertar o pH do hidrolisado, até valores próximos da neutralidade, adequados à atividade microbiana (Mateo et al., 2013). O ajuste de pH é frequentemente utilizado em combinação com outros métodos, uma vez que por si só não é suficiente para a obtenção de um meio adequado para as fermentações (Carvalheiro, 2005).

O uso do carvão ativado tem demonstrado ser um dos métodos mais promissores para a redução de compostos fenólicos e de furanos totais (Mateo et al., 2013). Neste método, o hidrolisado pode ser tratado através da sua passagem numa coluna empacotada com carvão ou colocando-o em contacto com o carvão sob agitação (Carvalheiro, 2005). O aumento da concentração de carvão ativado permite uma redução significativa dos principais compostos fenólicos e derivados de furanos. Para além da remoção de compostos tóxicos tem também sido relatado a perda de cor e de volume de hidrolisados tratados com carvão ativado (Mateo et al., 2013). A Fig. 14 mostra o aspecto anterior e posterior do licor hemicelulósico de resíduos de esteva submetido ao tratamento com carvão ativado, evidenciando a perda de cor provocada por este método.

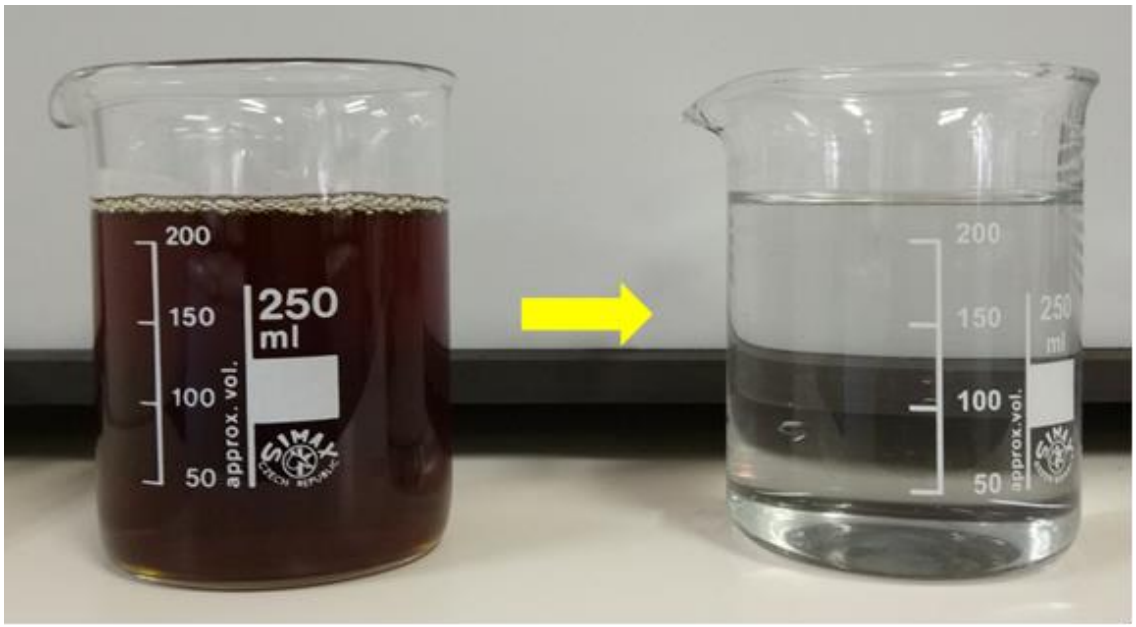

Fig. 14 Hidolisados hemicelulósicos de resíduos de destilaria de esteva tratados com carvão ativado (concentração 10%). Hidrolisado não tratado à esquerda; hidrolisado tratado à direita (Arquivo pessoal)

As resinas de troca iónica são um dos métodos mais eficientes para remoção de compostos inibidores dos hidrolisados hemicelulósicos antes da fermentação. Os hidrolisados podem ser tratados com diferentes tipos de resinas, nomeadamente aniónicas, catiónicas e resinas mistas, sendo necessária a abordagem sistemática para determinar o tipo de resina que poderá ser mais eficiente (Nilvebrant et al., 2001). Os processos de troca iónica têm demonstrado, inclusive, maior eficiência que o tratamento com carvão ativado na remoção de inibidores, como fenóis, derivados de furano, ácidos alifáticos e metais (Villarreal et al., 2006; Almeida et al., 2009), sendo as resinas aniónicas referidas por alguns autores como as mais eficientes na remoção desses diversos grupos de compostos (Larsson et al., 1999; Nilvebrant et al., 2001).

Para além dos anteriores, destacam-se também os estudos recentes de destoxificação com membranas (nanofiltração) (Santos et al., 2011, Morais et al., 2013), cuja especificidade relativamente a outros métodos físicos e químicos usuais é uma das suas principais vantagens. A aplicação de processos combinados (evaporação sob vácuo, tratamento com lacases, aumento de pH, tratamento com bissulfito, micro-arejamento) para a destoxificação de hidrolisados tem sido estudada com grande sucesso, alcançando remoções completas dos compostos inibidores (Geddes et al., 2015). Todavia, é necessário sublinhar as perdas de açúcares reportadas nos diferentes métodos de destoxificação, sendo esta uma das grandes desvantagens dessas abordagens (Mohagheghi, et al., 2006; Kamal et al., 2011; Mateo et al., 2013).

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CAPÍTULO II

Potential use of rockrose as a feedstock for biorefineries: a review

Júnia Alves-Ferreira, Helena Pereira, Maria C. Fernandes, Florbela Carvalheiro (2019). Potential use of rockrose as a feedstock for biorefineries: a review (em submissão)

Abstract

Cistus ladanifer is a relevant shrub species in the Mediterranean region. It produces an aromatic exudate known as labdanum which has been used for various applications in traditional medicines, i.e., as sedative, hemostatic, antiseptic, adstringent, tonic, expectorant, among others. *Cistus* essential oils are used in the perfume and cosmetic industries and are also known for their antioxidant and antimicrobial properties. Likewise, extracts of *Cistus ladanifer* contain a variety of volatile molecules and phenolic compounds that present a wide range of pharmacological activity. The use these species in the animal feed or in phytostabilization of mining areas also have been successfully employed. On the other hand, the lignocellulosic biomass, namely, lignin and polysaccharides from *Cistus* residues can be an interesting alternative to obtaining of high-value products in a biorefinery framework.

Keywords: *Cistus ladanifer*, Essential oils, Extractives, Bioactivity, Bioproducts

1. Introduction

Cistus ladanifer (crimson-spot rockrose) is a wild perennial shrub species which belongs to the Cistaceae family and to the *Cistus* genus that is mainly distributed in the Mediterranean countries such as France, Greece, Spain, Portugal, Morocco, Algeria and Cyprus (Heywood, 1985; Talavera et al., 1993; Weyerstahl et al., 1998; Ferreira et al. 2008; Morales-Soto et al., 2015).

The *Cistus ladanifer* species includes three subspecies: *ladanifer*, *africanus* (Dans) and *sulcatus* (Demoly). The subsp. *ladanifer* is mainly distributed in the Iberian Peninsula, France and northern Africa; the subsp. *sulcatus* is endemic to south-western Portugal; and the subsp. *africanus* is spread in southern Spain but more commonly found in northern Africa (Guzman & Vargas, 2009).

C. ladanifer is a shrub, 50 to 200 cm tall, generally erect, with a reddish-brown stem, hard wood and sticky bark. The 40-80 cm long leaves are impregnated by the labdanum, which makes them sticky and smelling strong (Demoly and Monserrat, 1993). It is considered a species of rapid growth and development that reproduces easily (Borges, 1989).

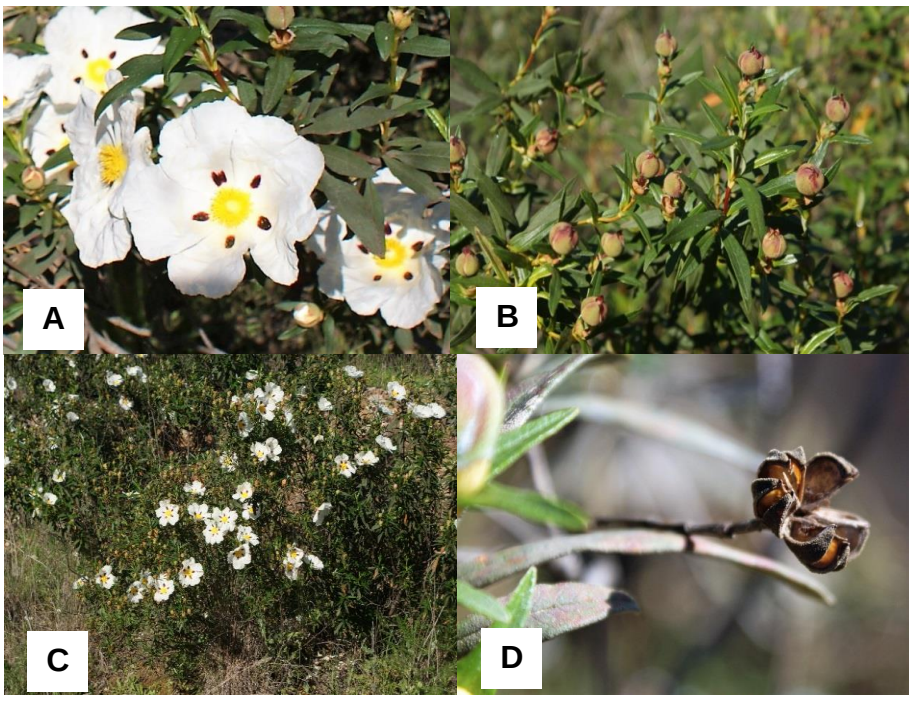

Fig. 1 *Cistus ladanifer* L.: A, flowers; B, flower buds; C, whole plant; D, open cysts

Cistus ladanifer occurs in a wide range of altitude, latitude, climates and soil types, but prefers acid and siliceous soils (Kidd et al., 2004). It has high-stress tolerance

and consequently a competitive potential under various environmental conditions, including poor soils with low organic matter content, low pH and high concentrations of trace elements, as well as hydric stress and high temperature and solar radiation (Simões et al. 2008). This tolerance may be associated with the activity of different isoenzymes, namely superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) (Santos et al, 2011), which are the major enzymes involved in detoxification of active oxygen species (AOS) (Pang et al., 2003).

Cistus ladanifer demonstrates important adaptative mechanism in post-fire plant dynamics and its recovery after burning is faster when compared with other shrub species (e.g. *Erica. australis* and *Calluna. vulgaris*) (Calvo et al. 2005). *Cistus* species tend to recover by an autosuccession process in areas affected by cutting and burning, being dominant from the first or second year (Tarrega et al., 2001). Its strong heat resistance suggests that there is a break of the seed dormancy by the high temperatures during a fire e.g. areas of *Quercus* woodland degraded by fire were occupied by *C. ladanifer* (Talavera et al., 1993). A study under controlled conditions demonstrated an increase in germination of seeds preheated at 100 °C when compared to seeds stored at room temperature (Pérez-García, 1997). In general, *C. ladanifer* seeds and plantlets present rapid germination and growth (Nuñez, 1989).

The fruits are globular lignified capsules (cysts) which are able to produce 500 to 1000 seeds, and it is estimated that a single plant produces annually 250 thousand seeds (Gallego, 2006; Delgado et al., 2008). The mature fruits remain attached to the plant and when open they release the seeds gradually, allowing a short distance dissemination for a long time. The dispersion of the seeds starts in autumn (Talavera *et al.*, 1993). The seeds are small, allowing an easy penetration and accumulation in the soil, and have a stiff and impermeable cover important for their longevity; however, they contain few nutrient reserves, which requires a quick start for the photosynthesis process (Gallego, 2006). The number of valves (5-12) per fruit can vary between populations, between plants and within the same plant. Rockrose is a highly polymorphic plant and the only species of the Cistaceae family with a variable number of valves per fruit. This variation may be a result of natural selection, phenotypic plasticity and developmental instability of the plant (Narbona et al., 2010).

The rockrose flowers are white with a crimson spot in the base of the petals. In Morocco there are two color varieties of *C. ladanifer* var*. albiflorus* has white petals and *C. ladanifer* var*. maculatus* has petals with red stains at the base (Greche et al., 2009). The flowers are large (ca. 64 mm of diameter), appear during spring (March-May) and produce abundant pollen and nectar (Talavera et al., 1993; Gomes et al, 2005). Size and longevity

of the flowers positively influence the incidence of florivores, mainly ants and beetles (Teixido et al., 2011).

2. Products from rockrose

The rockrose plants have been widely used, over time, as an important resource for primary health care given the low cost, accessibility and the accumulated ancestral experience. The aerial parts of rockrose are used for production of extracts, obtaining exudates and essential oils of complex composition and pharmacological properties that make further application targeted investigation interesting (Lourenço et al., 2015). The traditional use of the rockrose has already contributed to the socioeconomic development of the rural communities, as well as to ecological and territorial management (Morgado et al., 2005). The *Cistus ladanifer* products are described in detail below.

2.1. Labdanum extracts

C. ladanifer produces a sticky resin or aromatic exudate known as labdanum which has been used as a sedative, hemostatic, antiseptic, adstringent, tonic, expectorant and balsamic compound (Quer, 2005; De Andres et al., 1999; Stübing and Peris, 1998). The labdanum has been studied mainly due to its interest for the perfumery industry. It is produced in the leaves and secreted by trichomes found in adaxial and abaxial parts, especially in young leaves (Borges, 1988). The subspecies *ladanifer* is the most important for obtaining the labdanum, since the other subspecies are not used for this purpose (Morgado et al., 2005).

There are reports of the presence of pollen of *Cistus ladanifer* and other Cistaceae species in 15th and 16th century cesspits in Belgium, suggesting the use of *Cistus* plants to reduce bad smell (Deforce, 2005). The labdanum of *C. ladanifer* has been also used as incense in Arabic countries (Papaefthimiou, et al., 2014).

The crude gum of labdanum is obtained by removing from the surface of plant parts waxes, resinous matter and oily materials by a treatment with hot alkaline water. Different products can be obtained from the crude labdanum: resinoid of labdanum, concrete and absolute of labdanum, and labdanum oil (Weyerstahl et al., 1998; Surburg & Panten, 2006; Greche et al., 2009). Resinoid of labdanum can be extracted from the crude gum by methanol, ethanol or toluene. The concrete is a viscous product with balsamic odor that can

be extracted using apolar solvents (hexane, for example) from the whole plant and the absolute is prepared from the concrete by extraction with polar solvents (e.g. alcohol) (Surburg & Panten, 2006; Greche et al., 2009).

The chemical analysis of *C. ladanifer* labdanum extracts showed high content of labd-14-ene-lfi,18-diol; labd-14-ene-8,13-diol; labda-8(20),13(16),14-triene; labd-12-ene-15,18 diol; 16-kaurene; labda-8,14-dienoic acid; labdanoic acid; labdenoic acid; labda-7,8-dienoic acid and 6-oxo cativic-acid. Concrete and absolute extracts have demonstrated interesting inhibitory efficiency on the growth of various strains (e.g. *E. coli, Bacillus megaterium, Staphylococcus aureus, Aspergillus niger, Botrytis cinerea; Mucor racemosus*) (Greche et al., 2009).

2.2. Essential oils

Essential oils are organic compounds responsible for the aroma and are involved in the defense mechanisms of several species (Pereira & Meireles, 2010). They can be synthesized by any plant organs and are stored in secretory cells, cavities, canals, epidermal cells or glandular trichomes. In general, density is lower than that of water, and they are soluble in lipids and in organic solvents. Essential oils are also known for their antimicrobial, analgesic, sedative, anti-inflammatory and spasmolytic properties. They also attract insects, favoring dispersion of seeds and pollens and at the same time repel other undesirable insects (Bakkali et al., 2008).

The main components of essential oils are hydrocarbons, especially terpenes, and oxygenated compounds, such as alcohols, aldehydes, esters, ketones, phenols and oxides. Most of the compounds are monoterpenes (corresponding to about 90%) followed by sesquiterpenes (Pereira & Meireles, 2010). In general, the commercial cistus oils have a high content of easily degradable monoterpenes that requires further processing by the perfumery industry to obtain better quality products (Gomes et al., 2005).

The methods to obtain essential oils from plants are hydrodistillation, steam distillation, solvent extraction and supercritical extraction (Adam, 2006). Nevertheless, any of these methods present advantages and disadvantages. Drawbacks of distillation are the time required and the loss of valuable water-soluble compounds. Solvent extraction is carried out at lower temperatures, but there is a co-extraction of non-volatile compounds that requires a cleaning step that may cause loss of important volatile components. Supercritical fluid extraction is an interesting alternative, once the operation conditions are adjusted towards extraction selectivity although the co-extraction of waxes is unavoidable, requiring their separation (Teixeira et al., 2007).

C. ladanifer is particularly interesting as a source of essential oils for the cosmetics and perfume industry (Gomes et al., 2005; Nuñez-Oliveira et al., 1995). The rockrose plants used in distillation consist of 2 to 5-year-old whole plants and although the collection can occur during all the year, the oil yield is better in the warmer seasons. The *C. ladanifer* oil yield is in the order of 45 mL per 100 kg of fresh material, with a market value of 4-10 euros per mL of essential oil.

Table 1 shows the major constituents detected in *C. ladanifer* essential oil from plants grown in different countries. The diversity observed in the chemical composition of *C. ladanifer* essential oil can be due to several factors such as climatic and soil variations, the stage of the vegetative cycle, seasonal factors, part of the plant analyzed, and the method used to obtain the essential oil, among others (Viuda-Martos et al., 2011).

Table 1. Chemical composition (%) of cistus essential oils from plants grown in different countries: Portugal (Gomes et al., 2005), Central Spain (Verdeguer et al., 2012), Corsica with plants from Spanish origin (Mariotti et al., 1997) and Eastern Morocco (Zidane et al., 2013)

Component	Portugal[a]	Central Spain[a]	Corsica /Spanish origin)[b]	Eastern Morocco[a]
Monoterpene hydrocarbons				
Tricyclene	-	-	-	2.7
α-Pinene	2.1	4.70	39	4.2
Camphene	0.3	0.64	2.1	15.5
Pinocarvone	1.1	-	0.9	
Limonene	-	0.37	1.7	
γ-Terpinene	-	0.10	0.4	3.8
α -Terpinene			0.1	1.8
p-cymenene	-	1.17	1.7	2.3
Oxygenated monoterpenes				
Bornyl acetate	1.6	7.03	3.1	
Terpinen-4-ol	1.0	6.37	1.1	6.3
α-Terpineol		2.20		1.2
trans-pinocarveol	2.1	20.00	1.9	
Borneol	0.7	-	0.8	11.1
Myrtenal	0.7	2.26	0.5	
cis-Pinocamphone		3.84		
2 (10)-Pinen-3-one		5.05		
Verbonene		0.85	0.3	0.8
Camphor		0.86		1.5

p-Mentha-1,5-dien-8-ol		4.78		
Sesquiterpene hydrocarbons				
Viridiflorene	1.3	0.41		
C15H26O sesquiterpene alcohol	6.0	-		
Cyclosativene		0.70	0.7	0.6
Aromadendrene		1.77		
Allo-aromadendrene	0.8		1.9	
α-Copaene		0.62	0.8	
α-Cubebene				2.2
δ-cadinene	1.0	-	0.8	6.4
Oxygenated sesquiterpenes				
Viridiflorol	17.4	13.59	11.8	2.8
Spathulenol	0.8	0.53	0.5	
Globulol	5.0		0.3	
Ledol	-	4.36	3.3	
Caryophyllene oxide	1.8			
Palustrol	-	0.50		
Others				
2,2,6-trimethylcyclohexanone	2.8	-	0.9	7.3
Phthalates				
Diethyl phthalate				2.9
Bis (2-ethylhexyl) phthalate				0.2
Material used for hydrodistillation	dry leaves and small ranches	fresh leaves	Leaves and stems	dry leaves

[a] Components identified using gas chromatography (GC) and/or gas chromatography/mass spectrometry (GC/MS); [b] components identified by C-NMR spectroscopy and GC

More than 400 compounds have already been detected in essential oil compositions of *C. ladanifer*, including those present in trace amounts (Gomes et al., 2005). It is noteworthy that the content of viridiflorol was high for *C. ladanifer* oil produced in countries such as Portugal, Spain and France, reaching 17.4, 13.6 and 11.8% respectively. *Cistus* oils from Portuguese plants present organoleptic advantages, because they are rich in amber-like compounds and have low content of monoterpenes (Gomes et al., 2005). There are differences between Portuguese varieties of *C. ladanifer* even if grown in the same place. Populations of *Cistus ladanifer* var. *maculatus* have higher concentrations of α-pinene, b-pinene, γ-terpinene, verbenone than populations of *C. ladanifer* var. *albiflorus* (Robles et al., 2003).

Verdeguer et al. (2012) observed that trans-pinocarveol (20%) was the main compound in *C. ladanifer* essential oil obtained from plants grown in Central Spain; viridiflorol (13.6%), bornyl acetate (7.0%) and terpinen-4-ol (6.4%) also presented

important concentrations, setting up an essential oil rich in oxygenated compounds. *C. ladanifer* grown in Corsica showed high percentages of α-pinene (39%), followed by viridiflorol (11.8%), ledol (3.3%) and bornyl acetate (3.1%). Viridiflorol was one of the main compounds identified in the oils of *C. ladanifer* grown in Portugal, Spain and France (11.8 –17.4%) while the main components from *C. ladanifer* from Morocco were camphene (15.5%) and borneol (11.1%) that presented small amounts in the samples of the other countries.

Viridiflorol and bornyl acetate are compounds which may be responsible for the antimicrobial power of the cistus oil against several strains (*E. coli, Bacillus megaterium, Staphylococcus aureus, Aspergillus niger, Botrytis cinerea; Mucor racemosus*) (Greche et al., 2009). The potential of cistus oil to inhibit or block the germination of weed species (e.g. *Amaranthus hybridus, Portulaca oleracea, Chenopodium album, Conyza canadensis* and *Parietaria Judaica*) was also demonstrated in vitro conditions (Verdeguer et al., 2011).

Zidane et al. (2013) detected as the major compounds in cistus oil from Morocco camphene (15.5%), borneol (11.1%), cyclohexanol-2, 2, 6 tremethyl (7.3%), terpineol-4 (6.3%) and α-pinene (4.2%); these authors also found phthalates.

Phthalates are considered hazardous to human health (Peakall, 1975) and losses in the manufacturing processes and leaching out from material can contaminate the environment (Fromme et al., 2002). Removing of phthalates from essential oil and aqueous phase have been reported (Özer et al., 2012; Xiong et al., 2013).

Many other compounds occurred in small amounts in the essential oils of *C. ladanifer*. In fact, the odors and flavor of the essential oil result from the combination of all these components, including the trace components (Anitescu et al., 1997).

2.3. Extractives

Table 2 shows the major phytochemical categories of volatile and non-volatile compounds isolated from extracts of different parts of *C. ladanifer*. Chromatographic and spectroscopic tools have enabled the identification of various chemical groups with important biological activities. Exudates of *C. ladanifer* are rich in terpenoids, such as monoterpenes, sesquiterpenes, labdane-type, diterpenes, phenylpropanoids, including flavonoids, phenolics and tannins, and carbonylic compounds (Papaefthimiou, et al., 2014).

Table 2. Major phytochemical constituents of *Cistus ladanifer* extracts

Compound	Chemical group	Part of the plant	References
Volatiles compounds			
α-Pinene	***Monoterpene hydrocarbons***	Aerial part; shoots	Morales-Soto et al., 2015; Santos et al., 2016
Camphene		Aerial part; shoots	Morales-Soto et al., 2015; Santos et al., 2016
Pinocarvone		Aerial part; shoots	Morales-Soto et al., 2015; Santos et al., 2016
Limonene		Aerial part	Morales-Soto et al., 2015
α-Phellandrene		Leaves	Ramalho et al., 2009
γ-Terpinene		Aerial part	Morales-Soto et al., 2015
α -Thujene		Aerial part	Morales-Soto et al., 2015
p-cymene		Aerial part	Morales-Soto et al., 2015
Bornyl acetate	***Oxygenated monoterpenes***	Aerial part; shoots	Morales-Soto et al., 2015; Santos et al., 2016
Terpinen-4-ol		Aerial part; shoots	Morales-Soto et al., 2015; Santos et al., 2016
α-Campholenal		Aerial part	Morales-Soto et al., 2015
trans-pinocarveol		Aerial part	Morales-Soto et al., 2015
Borneol		Leaves; aerial part	Ramalho et al., 2009; Morales-Soto et al., 2015
Myrtenal		Aerial part	Morales-Soto et al., 2015
(cis)-Verbenol		Leaves; shoots	Ramalho et al., 2009; Santos et al., 2016
Verbonene		Leaves; aerial part; shoots	Ramalho et al., 2009; Morales-Soto et al., 2015; Santos et al., 2016
Camphor		Leaves; shoots	Ramalho et al., 2009; Santos et al., 2016
Viridiflorol		Shoots	Santos et al., 2016
Globulol	***Oxygenated sesquiterpenes***	Shoots	Santos et al., 2016
Ledol		Leaves	Ramalho et al., 2009
Caryophyllene oxide		Shoots	Santos et al., 2016
Eugenol	***Phenylpropene***	Leaves	Ramalho et al., 2009
Benzenepropanoic acid	***Phenylpropanoid***	Shoots	Santos et al., 2016
2-Phenylethanol	***Alcohol***	Leaves; aerial part	Ramalho et al., 2009; Morales-Soto et al., 2015
Acetophenone	***Aromatic ketone***	Leaves	Ramalho et al., 2009
Thuja-2,4(10)-diene	***Others***	Aerial part	Morales-Soto et al., 2015
Rhododendrol		Shoots	Santos et al., 2016
2,2,6-trimethylcyclohexanone		Leaves; aerial part; shoots	Ramalho et al., 2009; Morales-Soto et al., 2015; Santos et al., 2016
Soluble compounds (phenolics)			
Apigenin	***Flavonoids***	Leaves; aerial part; whole plant	Chaves et al., 1998; Fernández-Arroyo et al., 2010; Tomas-Menor et al., 2013
Apigenin-6-C-glucose-8-C-glucose		Leaves	Barros et al., 2013
Apigenin metilether		Whole plant	Tomas-Menor et al., 2013
Kaempferol dimethylether		Aerial part; leaves; whole plant	Fernández-Arroyo et al., 2010; Barros et al., 2013, Tomas-Menor et al., 2013

Kaempferol diglycoside		Whole plant	Tomas-Menor et al., 2013
4´(o)methyl-apigenin		Leaves	Chaves et al., 1998
7(*o*)methyl-apigenin		Leaves	Chaves et al., 1998
3-methyl-kaempferol		Leaves	Chaves et al., 1998
4´-dimethyl-kaempferol		Leaves	Chaves et al., 1998
3,7-dimethyl-kaempferol		Leaves	Chaves et al., 1998
3,7,4´-trimethyl-kaempferol		Leaves	Chaves et al., 1998
Kaempferol methylether		Aerial part; leaves	Fernández-Arroyo et al., 2010; Barros et al., 2013
Quercetin-O-hexoside-Ohexoside		leaves	Barros et al., 2013
Epigallocatechin		Aerial part; leaves	Fernández-Arroyo et al., 2010; Barros et al., 2013
Gallic acid	***Phenolic acids and derivatives***	Aerial part	Fernández-Arroyo et al., 2010
Glucogallin (isomer)		Aerial part	Fernández-Arroyo et al., 2010
Gentisoyl glucoside		Aerial part; whole plant	Fernández-Arroyo et al., 2010; Tomas-Menor et al., 2013
Digaloil-*β*-D-glucopiranose		Aerial part	Fernández-Arroyo et al., 2010
Galloyl glucose		Leaves	Barros et al., 2013
Mirciaphenone B		Aerial part	Fernández-Arroyo et al., 2010
Punicalagin isomer 1	***Ellagic acid and derivatives***	Aerial part, leaves	Fernández-Arroyo et al., 2010; Barros et al., 2013
Punicalagin isomer 2		Aerial part, leaves	Fernández-Arroyo et al., 2010; Barros et al., 2013
Punicalagin gallate 1		Leaves	Barros et al., 2013
Punicalagin gallate 2		Leaves	Barros et al., 2013
Punicalin		Aerial part; whole plant	Fernández-Arroyo et al., 2010; Tomas-Menor et al., 2013
Cornusiin		Aerial part	Fernández-Arroyo et al., 2010
Ellagic acid-7-xyloside		Aerial part	Fernández-Arroyo et al., 2010
Ellagic acid		Aerial part	Fernández-Arroyo et al., 2010
Ducheside A		Aerial part	Fernández-Arroyo et al., 2010
Shikimic acid	***Others***	Aerial part Whole plant	Fernández-Arroyo et al., 2010; Tomas-Menor et al., 2013
Quinic acid		Aerial part Whole plant	Fernández-Arroyo et al., 2010; Tomas-Menor et al., 2013
Hexahydroxydiphenoyl-D-glucose (isomer)		Aerial part	Fernández-Arroyo et al., 2010
Phenethyl-β-primeveroside		Aerial part	Fernández-Arroyo et al., 2010

Flavonoids and diterpenes (C20-carbon) are main secondary metabolites and can contribute to a variety of functions (Pascual et al, 1984; Fernández-Arroyo et al. 2009), as defense purposes and are also precursors of hormones such as tocopherols and gibberellins. Monoterpenes (C10-carbon) and sesquiterpenes (C15-carbon) are volatile compounds that contribute to plant odors and are repellants for herbivores, respectively (Papaefthimiou, et al., 2014). Flavonoids may vary according to season, since their synthesis is induced by climatic factors (Sosa et al., 2005). Flavonoids such as apigenin, 4'-methyl-apigenin, 7-methyl-apigenin, 7,4'-dimethyl-apigenin, 3-methyl-kaempferol, 3,4-dimethyl-kaempferol, 3,7-dimethyl-kaempferol and 3,7,49-trimethyl-kaempferol can be found in the leaf resin (Chaves et al., 1998). Phenolic compounds such as ferulic acid, p-hydroxybenzoic, vanillic, p-coumaric and caffeic acids, in association with terpenes (α and b-pinene) have been detected not only in aqueous extracts, but also on soil samples occupied by *C. ladanifer* (Herranz et al., 2006). The ellagitannins, in particular punicalagins derivatives, are the main compounds found in the

aqueous extract of the aerial part of *C. ladanifer*, with values of 0.24% (w/w) for gallic acid, and 3:50% (w/w) for all ellagitannins. Tannin content has a significant proportion of 6.8% of total dry matter (Barrajón-Catalán et al., 2010).

Diterpenes and flavonoids contents vary seasonally. The maximum concentration of diterpenes in the leaves occurs in winter and the minimum in spring-summer, but the maximum production of flavonoids is detected in summer and the minimum in winter. Low temperatures increase the amount of diterpenes and higher temperatures increase the concentration of flavonoids (Alías et al., 2012). Flavonoids are systematically lower in plants grown in shaded areas than in those grown in open areas. These compounds protect the plants from the harmful effects of UV radiation, and irradiation is the main inductor in the production of flavonoids. This induction may be synergistically increased by drought, where there is a higher production of methylated flavonoids (kaempferols and 7-methylated apigenins), suggesting that the methylated form is part of the defense mechanism of the plant against hydric stress (Chaves et al., 1997). Seasonal variation in the levels of phenolic compounds and condensed tannins have also been reported by Guerreiro et al. (2016). The accumulation of phenolic substances in various tissues and their deposition in cortical cells defend the plants from predators and protect the internal tissues against UV-B radiation, respectively (Micco & Arone, 2007).

Vitamins, reducing sugars and polyunsaturated fatty acids were also detected in rockrose. *C. ladanifer* leaves presented a very high level of ascorbic acid (647.6 mg/g dry weight) and of sugars such as fructose, glucose, sucrose and raffinose, being fructose the most abundant (40.3 mg/g). Fatty acids as eicosadienoic acid, arachidic acid and linolenic acid were identified in cistus extracts (Guimarães et al., 2009). The highest concentration of polyunsaturated fatty acids (PUFA) occurs in winter and spring and of branched chain fatty acids (BCFA) in summer (Guerreiro et al., 2015).

3. Bioactivity

Various compounds from different phytochemical groups identified in rockrose are associated to different biological activities (Table 3) that may be of interest for the pharmaceutical and food industries (Pereira et al., 2010). Among the pharmaceutical properties the antibacterial, antiviral, antioxidant activities are highlighted.

Table 3. Biological activities of *Cistus ladanifer* extracts

Plant Part	Extract	Biological activities	References
fresh leaves from flowering stems	methanol/water extract	antifungal	Barros et al., 2013
leaves	aqueous extract	autotoxicity	Sosa et al., 2006
wood/stalks, bark and leaves	ethanol extract and acetone extract	antioxidant activity	Andrade et al., 2009
leaves	aqueous extracts	allelopathic	Herranz et al., 2006
aerial part	aqueous extract	antihypertensive	Belmokhtar et al., 2009
/Whole plant	hydroalcoholic and spray-dried/ spray-dried aqueous extract	antibacterial	Tomas-Menor et al., 2013
leaves	aqueous extract	antioxidant antimicrobial, cytotoxic activity against human cancer cells	Barrajón-Catalán et al., 2010
leaves	*Flavonoids extract*	allelopathic	Chaves et al., 2001
shoots	water soluble and volatile compounds	phytotoxic	Dias & Moreira, 2002
Leaves and small branches whole plant	essential oil Labdanum extracts	Antifungal antibacterial	Greche et al., 2009
aerial part	essential oil	herbicidal activity	Verdeguer et al., 2012
fruits, stems, flowers and leaves	essential oil water, ethanol, ethanol: water (50:50), methanol, methanol: water (50:50), acetonitrile	antioxidant	Zidane et al., 2013

3.1. Antimicrobial activity

C. ladanifer is known for its antimicrobial properties due to the phenolic compounds that are present in the plant exudate (Sánchez-Hernández et al., 2002). Antifungal effects against species responsible for a series of diseases such as *C. glabrata*, *C. parapsilosis* and *C. albicans* (MIC < 0.05 mg/mL) were observed in rockrose phenolic extracts containing phenolic acids and their derivatives, ellagic acid derivatives and flavonoids (catechins, flavonols and flavones) (Barros et al., 2013). *Cistus* extracts can be considered as good antibacterial agents, in particular against gram-positive bacteria (e.g. *Staphylococcus aureus*). This fact might be due to the combination of ellagitannins present in the extracts (Barrajón-Catalan et al., 2010).

One study, using hydroalcoholic and aqueous extracts obtained with different drying methods, reports that the hydroalcoholic extracts exhibit strong antibacterial activity and higher levels of polyphenolic compounds compared to freeze-dried aqueous extracts. One explanation could be the degradation of some polar compounds during the preparation of the extract that contribute to inhibition of gram-positive bacteria (e.g. cyclohexane carboxylic acids, hexa-hydroxydiphenyl glucose, gallotannins, punicalin and epigallocatechin). On the other hand, the inhibitory activity against gram negative bacteria

is associated to the presence of galloylated flavonols and some flavonols specific (Tomás-Menor et al., 2013).

3.2. Antioxidant activity

Several methods have been applied to prove the antioxidant power of the essential oil and also of different extracts *C. ladanifer*, among which: DPPH and ABTS radical scavenging capacity assay, oxygen radical absorbance capacity (ORAC) assay, superoxide dismutase (SOD) assay, and ferric reducing antioxidant potential (FRAP) assay (Andrade et al., 2009; Barrajón-Catalán et al., 2010; Dudonné et al., 2009; Guimarães et al., 2010; Zidane et al., 2013). In general, these results revealed a significant antioxidant activity of *C. ladanifer*. Ethanolic extract, for example, has displayed radical scavenging activity about 2 times higher than that of Trolox (antioxidant standard) (Andrade et al., 2009). Aqueous from leaves showed 5% of DPPH inhibition and 27 % of ABTS inhibition (Dudonné et al., 2009). Different extracts from different *C. ladanifer* parts exhibited a higher scavenging ability of DPPH radicals when compared to essential oils of *C. ladanifer* (Zidane et al., 2013).

However, there is agreement that additional studies are needed in order to identify the chemical compounds these extracts that contribute to the antioxidant activities and thus better understand their mechanisms as radical scavengers (Andrade et al., 2009; Barrajón-Catalán et al., 2010).

3.3. Allelopathic activity

The allelopathic ability of the phytotoxic compounds present in *C. ladanifer* exudate is associated to a decreased richness and diversity in species that share the same habitat, such as *P. angustifolia*, *P. latifolia*, *R. alaternus*, *H. ocymoides*, *populifolius C.*, *Erysimum lagascae*, *Brassica area barrelieri, Silene tridentata*, and *Moricandia moricandioides* (Herranz et al., 2006; Gallego et al. 2008). The direct incorporation of phenolic compounds into the soil occurs through the falling of the leaves. These compounds have high persistence (ca. 10 months) and although present in low levels in the order of mg/g, their negative effect on regeneration was observed.

The auto-allelopathic potential of *C. ladanifer* was also demonstrated by using an aqueous solution from leaves for inhibiting the germination and cotyledon emergence of its own seeds (Alías et al., 2006).

3.4. Antihypertensive activity

A pharmacological study on *C. ladanifer* aqueous extracts demonstrated their antihypertensive effect. Aqueous leaves extract of cistus (500 mg/kg/day - oral administration) reduced systemic blood pressure of two models of experimental hypertension, the L-NAME and renovascular 2K-1C, acting in preventive and curative manner. A reversal of the endothelial disfunction in both animal models was also observed. However, further studies are needed to identify the chemical compounds involved in the process (Belmokhtar et al., 2009).

3.5. Antitumoral activity

Cistus extracts were able of inhibit the proliferation of pancreatic cancer cells M220 and MCF7/HER2 and JIMT-1 breast cancer cells but this cytotoxic potential on cancer cells merits further investigation to determine its potential mechanism (Barrajón-Catalán et al., 2010).

Flavonoids are generally nontoxic and associated to diverse biological activities among which prevention of cancer. Some epidemiological studies have associated the high dietary intake of flavonoids present in fruits and vegetables to low cancer prevalence in humans; *in vivo* and *in vitro* assays demonstrated action in carcinogen inactivation, antiproliferation, cell cycle arrest, induction of apoptosis and differentiation, inhibition of angiogenesis, antioxidation and reversal of multidrug resistance or a combination of these mechanisms (Ren et al., 2003).

3.6. Hypoglycemic and hypolipidemic activities

Treatment using cistus aqueous extract was applied in diabetic rats. The dose of 500 mg/kg body weight administered orally for a period of 28 days occasioned a reduction in the levels of blood glucose, alanine aminotransferase, aspartate aminotransferase, urea and creatinine in the animals. Besides having improved glucose tolerance in diabetic rats, the aqueous extract of *C. ladanifer* reduced total cholesterol, triglycerides and low-density lipoprotein-cholesterol levels (El Kabbaoui et al., 2016)

4. Potential uses

Besides the traditional use of rockrose extracts and their already well-established use in the perfumery industry as a fixative of perfumes, other potential applications have been attributed to *C ladanifer*. Among these, the use of the plant with nutritional benefits for animal health, the phytoremediation of soils contaminated by heavy metals and the use as raw material for the production of bioethanol are some of the new future directions given to the valorization of the rockrose as a commercial plant.

4.1. Phytoremediation

Phytoremediation is an emerging environmentally friendly and low-cost technology that uses plants and their associated microorganisms to remove pollutants from contaminated sites, especially heavy metals (Hooda, 2007). *C. ladanifer* was reported as able to survive and grow in soils with high concentrations of toxic elements such as Mn, Cu, Zn and Pb, As (Alvarenga et al., 2004; Abreu et al., 2011; Santos et al., 2012). Observations from mining areas suggest selectivity in absorption and translocation of metals. Therefore, the tolerant behavior of this species in soils with toxic elements can expand the possibilities of phytoremediation strategies for environments with significant rates of contamination (Alvarenga et al., 2004).

Another study on the rockrose resistance to heavy metals was conducted in hydroponic experiments. Higher tolerance to metals such as Cd, Co, Cr, Mn and Ni was observed in plant populations originating from ultramafic soils or soils developed on basic rock, while populations originating from acid-rock soils exhibited higher tolerance to Cu and Zn. This species presents different patterns in the accumulation of metals: Cd, Co, and Mn accumulated in the aerial part while Cu and Pb were not transported efficiently through the roots up to the shoots. In general, *C. ladanifer* is able to accumulate heavy metals in the aerial parts without inhibiting plant growth (Kidd et al., 2004).

Thus, its potential for phytostabilization mines soil can be considered suitable taking into account trace element concentrations in leaves and seeds and seed germination rates (Santos et al., 2012). Beyond immobilization of chemical elements, the phytostabilization with autochthonous species also increase organic matter and water retention capacity, improving soil structure and reducing erosion (Abreu & Magalhães, 2009).

It is worth mentioning that hexane extracts from *C. ladanifer* plants growing in mining areas did not exhibit potentially hazardous elements, suggesting no human health risk (Santos et al., 2016). Therefore, the high resistance of *C. ladanifer* to nutrient

unbalanced soils, with potential toxic elements and adverse climatic conditions make this species appropriate for phytoremediation and revegetation of contaminated soil (Rossini-Oliva et al., 2016).

4.2. Animal feed

The use *of C. ladanifer* as a food supplement for animal nutrition and productivity increase presented interesting results. Inclusion of leaves and soft stems of *C. ladanifer* in diets of lambs supplemented with an oil blend (sunflower and linseed oils) showed a weight gain and an increase in subcutaneous fat deposition (11.3 *vs* 9.2%) when compared to other diets, thus reinforcing its use for improvement of animal diet (Jerónimo et al, 2010). The fatty acids provided by the diet with *C. ladanifer* benefit the health and do not jeopardize animal performance (Jerónimo et al, 2010).

Another approach using a *C. ladanifer* diet, with or without oil supplementation, reduced lipid oxidation of lamb meat in pro-oxidant conditions and did not affect the meat sensory properties. These observations suggest that the use of *C. ladanifer* can be an alternative in ruminant feeding (Jerónimo et al., 2012).

Incorporation of ethanolic *C. ladanifer* extracts in rabbit feed showed also to be possible, since it did not affect the productivity, although the consumption rate was higher due to excess fiber and low protein (Zamora-Lozano et al., 1984). Phenolic crude extracts from *C. ladanifer* were also employed in the treatment of soybean meal to reduce rumen degradation that may be advantageous to increase the flux of potential feed protein into the post-ruminal compartments (Dentinho et al., 2014).

4.3. Bioethanol production

Lignocellulosic biomass is an abundant and inexpensive feedstock that can be used to produce bioethanol. Lignocellulose materials are frequently hydrolyzed by treatment with acids and subsequently fermented by microorganisms for bioethanol production (Balat, 2011).

Some studies reported rockrose residues as a potential source for bioethanol production. Gil et al. (2012) demonstrated that a dilute acid pretreatment was effective for carbohydrate solubilization from *C. ladanifer* residues achieving a maximum concentration of 302.2 mg/g of total sugars. The subsequent enzymatic hydrolysis of the solid fractions resulting from the acid hydrolysis pretreatment was enhanced and varied with temperature, cellulase concentration and incubation time (Ferreira et al., 2009.).

Steam explosion followed by alkaline extraction was also an effective pretreatment of rockrose residues leading to a 75% higher glucose yield than the untreated raw material. The use of simultaneous saccharification and fermentation (SSF) for bioethanol production reached a yield of 22.1 g bioethanol/100 g of rockrose residues and proved more efficient than separate enzymatic hydrolysis and fermentation (SHF) (Ferro et al., 2015).

5. Future perspectives

The most companies processing *C. ladanifer* for the cosmetics and perfume industry are usually small their overall potential is often not explored. Nevertheless, there are important companies in countries such as Japan, Netherlands, France, and especially in Spain, where this species extends to a total of 2.106.717 ha in shrub formations and under the sparse forest (Morgado et al., 2005; Pérez et al., 2011).

In general, the residues resulting from the extraction process (steam distillation or solvent extraction) are used only for feed the boilers or for energy production by combustion, a very low-value application. The valorization of this extracted biomass would improve the overall economy of the process since the plants contain only low amounts of the main compounds of interest (usually essential oils represent ≤ 1% and labdanum gum ≤ 6%). As the extraction processes are relatively soft, the solid residues remain largely unaltered and have potential to be used in the production of a wide range of other products. Indeed, the chemical composition of the residues, in particular, their extractives, polysaccharides and lignin are favorable for the valorization in a biorefinery framework (Alves-Ferreira et al., 2017; 2019). This would constitute an opportunity for these industrial units, which are now focused only on e labdanum gum and essential oil, thus opening the possibility to produce novel products. Fig. 2 presents a scheme of integration of processes and products for the valorization of the *Cistus ladanifer* in a biorefinery framework, where the plant and their residues can be fully exploited to obtain added-value products or other applications.

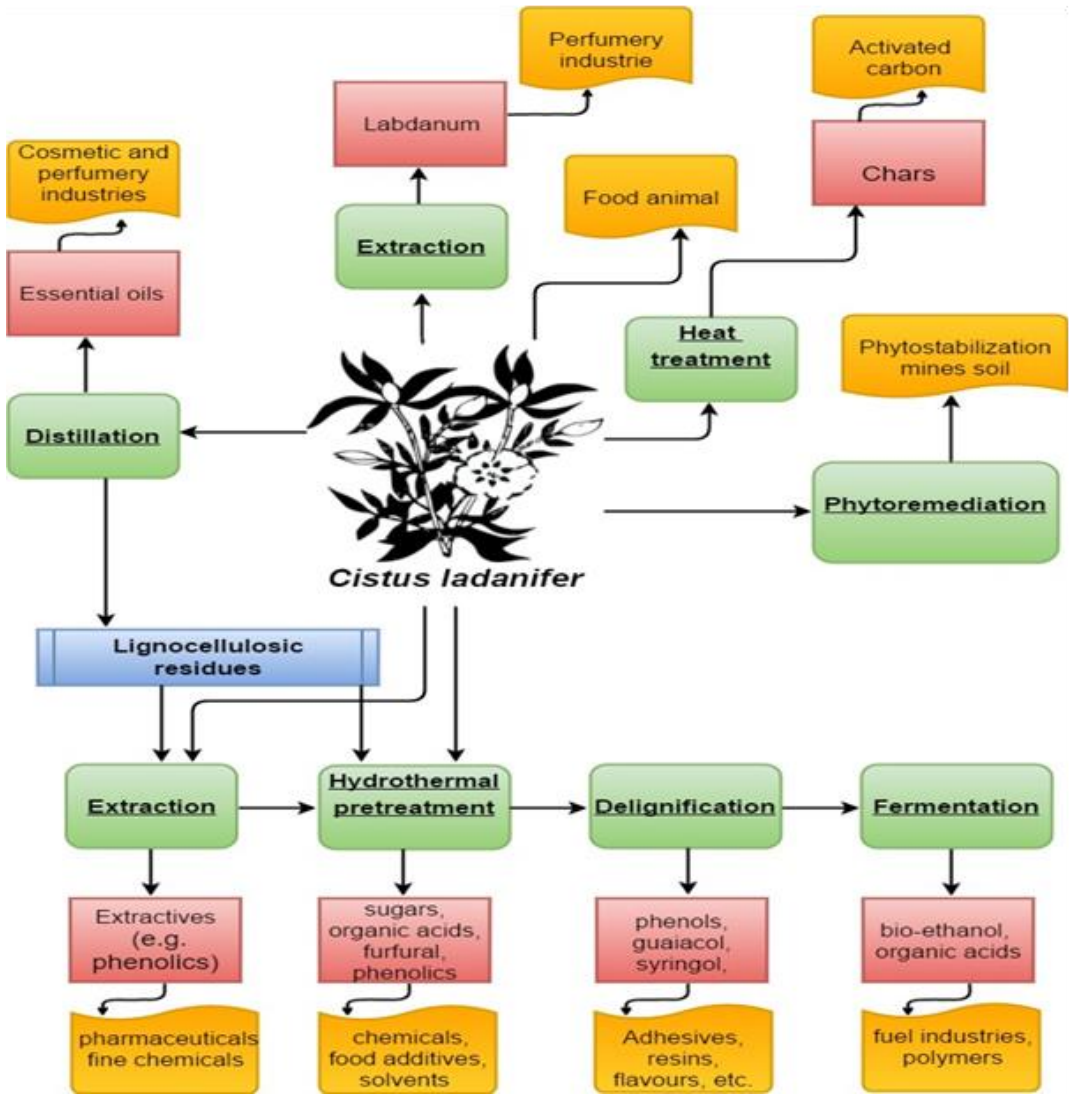

Fig. 2 Scheme of potential use of *Cistus ladanifer*, with emphasis on its integrated valorization in a biorefinery framework for obtaining of added-value products

In a biorefinery, the biomass is used integrally in a sustainable way for concomitant production of biofuels, energy, materials, and chemicals, preferably with added value (Carvalheiro et al, 2008; SIADEB, 2011). The chemical use of the lignocellulosic biomass requires the fractionating of the material, often called pretreatment processes. Deconstruction processes of the biomass cellular matrix allow to obtain high quality hemicelluloses, cellulose and lignin derived products. Fractionation methods include physical, chemical and biological processes. However the processes have to be optimized for the specific biomass raw material and should exploit the different properties of lignocellulosic materials (Carvalheiro et al., 2013). Their integration is also an important aspect for the economic viability of any biomass-based transformation aiming at minimizing energy consumption and waste.

In this context, fractionation processes could be applied to the *Cistus ladanifer* residues from the distilleries, as well as to plants harvested directly from the field. However, the use of residues is more advantageous, since the essential oils and labdanum have already an established market. Future investigations addressing the use of distillery residues in animal feed may be important to demonstrate if there is also a potential valorization by this route.

In addition, extractive fractions can be also targeted as potential valuable chemicals namely bioactive products, providing another pathway for the use of this species. The products obtained from *Cistus ladanifer* extraction have a varied composition and the determination of the compounds responsible for the bioactivity becomes complex; furthermore, the synergistic action of compounds seems to outweigh the isolated action of each constituent (Lourenço et al., 2015). Therefore, additional studies are needed to determine which compounds are responsible for the pharmacological activities, as well as the mechanisms of action involved, toxicity, possible interactions and secondary effects (Lourenço et al., 2015).

Additional studies using *Cistus ladanifer* as lignocellulosic material are also necessary for a greater knowledge of this species and to exploit the different potential pathways of valorization. The development and optimization of integrated processes for the use of *C. ladanifer* resources in a biorefinery framework is particularly relevant for the valorization of the endogenous biomass in small and large scales.

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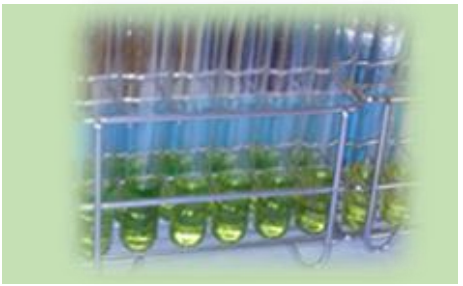
CAPÍTULO III



Cistus ladanifer as a source of chemicals: structural and chemical characterization

A informação constante neste artigo faz parte da seguinte publicação:

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Abstract

Different biomass fractions of *Cistus ladanifer*, and solid residues from essential oil distilleries were structurally and chemically evaluated. The *C. ladanifer* biomass fractions showed chemical differences mainly related to extractives (e.g. 10.8% and 53.7% in stems and leaves) and lignin (e.g. 21.2% and 15.4% in stems leaves). The distillery residues were characterized by 41.5% extractives and 19.3% lignin; and polysaccharide glucose 51.7% and xylose 24.9% of total monosaccharides. The polar extracts had a high content in phenolics and revealed high antioxidant activity (IC_{50} 3.2 and 4.7 µg/mL in stems and cysts extracts).

The lignin structure showed a predominance of S units in the stem (H:G:S of 1:25:50) and a balanced proportion of H, G and S-units in leaves (H:G:S of 1:1.4:1).

The characteristics of *C. ladanifer* biomass allow several routes of valorization. The high extractive contents point out to the potential use as a source of phytochemicals by applying extraction procedures while the remaining lignocellulosic material after extraction may be directed towards lignin and carbohydrates applications. The use of *C. ladanifer* biomass for an extractives-lignocellulosic-based biorefinery therefore represents a potential valorization that may contribute to an additional revenue for the present essential oil distilleries.

Keywords: Extractives, Antioxidant activity, Polysaccharides, Lignin, Biorefinery

1. Introduction

Most genus of the Cistaceae family, including shrubs, semi-shrubs and herbs, are distributed in the Mediterranean region where they occur especially in open areas and poor soils (Gusmán & Vargas, 2009). In particular, the genus *Cistus* of dicotyledonous perennial herbaceous plants is wide spread and includes some species whose extracts have been used in folk medicine and as fragrances (Papaefthimiou et al., 2014). *Cistus ladanifer* (CL) is one of the main species producing labdane, a resin employed as a natural fixative and as fragrance for composing amber and leathery notes, and also used as incense (Gomes et al., 2005; Papaefthimiou et al., 2014). Other odoriferous material may also be obtained from fresh leaves and branches: essential oil by steam distillation, cistus concrete by nonpolar solvent extraction and absolute by taking up concrete. These products are used in perfumery and come mainly from Spain (Weyerstahl et al., 1998; Surburg and Panten, 2006; Greche et al., 2009).

The solid residues generated from the CL essential oil extraction process cannot be reused for gum extraction and vice-versa but they constitute a lignocellulosic material that may be valorized in different ways, namely as a source of other extractives. In fact, the use of biomass within the biorefinery concept has seen a growing interest since it allows producing a rich combination of products including biofuels, pulp and paper, biomaterials and biochemicals. Residues such as bark and foliage are rich in phytochemicals that can be used as biopharmaceuticals, food additives and nutraceuticals, biopesticides and cosmetics, although they still remain under-exploited (Devappa et al., 2015; Miranda et al., 2016; Sartori et al., 2018).

Several pharmacologically interesting compounds were already identified in *C. ladanifer* fractions e.g. monoterpenes, sesquiterpenes and labdane-type diterpenes, flavonoids, phenolics, tannins and carbonylic compounds (Papaefthimiou et al., 2014). The different extracts showed various biological properties: antioxidant (Andrade et al., 2009; Barrajón-Catalán et al., 2010; Zidane et al., 2013), antibacterial (Greche et al., 2009; Tomas-Menor et al., 2013), antifungal (Greche et al., 2009; Barros et al., 2013), cytotoxic (Barrajón-Catalán et al., 2010), allelopathic (Chaves et al., 2000; Herranz et al., 2006), antihypertensive (Belmokhtar et al., 2009) and hypoglycemic (Kabbaoui et al., 2016). Only some works reported recently on the potential use of the *C. ladanifer* lignocellulosic material for obtaining carbohydrates and lignin-derived products, and extractive fractions targeted as potential valuable chemicals within a biorefinery platform (Gil et al., 2012; Ferro et al., 2015; Alves-Ferreira et al., 2017).

It is acknowledged that the specific chemical and structural composition of the different biomass fractions is important to design the various valorization pathways. This

paper addresses this issue by dividing the *C. ladanifer* plant into stem, branches, leaves and cysts, and characterizing them, as well as the distillery residues obtained from two industries in Portugal. The chemical summative composition, the monomeric composition of polysaccharides and lignin, and the content, composition and bioactivity of extractable compounds were studied as well as the anatomical structure of the stem. The objective is to characterize in detail the chemical and structural features of *C. ladanifer* and of its residues obtained from the industrial essential oil distillation in order to provide background data for their integration in biorefineries.

2. Materials and methods

2.1. Sampling

Cistus ladanifer plants with 2 to 5 years of age were randomly selected and harvested from Quinta Essência (Portel, Portugal). These plants were fractionated into stem, branches, leaves and cysts, and air dried under well ventilated conditions during 15 days. The *C. ladanifer* residues obtained after steam distillation (CLR) for extraction of essential oils were collected from two distillery units: Quinta Essência (Portel, Portugal) (CLR1) and SILVAPOR - Ambiente & Inovação, Lda., Quinta da Devesa (Idanha-a-Nova, Portugal) (CLR2). The plants used for production of essential oils by these distilleries were between 2 and 4 years of age. Stems, branches, leaves, cysts and CLR 1 and CLR2 were ground with a knife mill with an output sieve of 6x6 mm^2. The chemical characterization was made on granulometric fraction 40-60 mesh (0.250-0.450 mm). The samples were stored in individual lots at room temperature.

2.2. Anatomical characterization

Stem samples from *C. ladanifer* of 2 to 5-year-old plants were impregnated with DP1500 polyethylene glycol and transverse sections of approximately 17 µm thickness were prepared with a Leica SM 2400 microtome using Tesafilm 106/4106 adhesive for sample retrieval. The sections were stained with a double staining of chrysodine/astra blue and mounted on Kaiser glycerin. After 24 h, the lamellas were submerged into xylol during 30 min to remove the Tesafilm, dehydrated in 96% and 100% ethanol and mounted in Eukitt. Stem bark and wood samples were also macerated in a 1:1 solution of 30% H_2O_2 and CH_3COOH at 60°C for 48 h and stained with safranine.

The microscopic observations were made with a Leica DMLA optical microscope and the photomicrographs were taken with a Nikon FXA camera.

2.3. Chemical characterization

The ground material was sieved in a vibratory apparatus, and the 40-60 mesh fraction (0.250-0.425 mm) was recovered and used for the chemical analysis. The summative chemical composition of the samples was analyzed by sequential determination of extractives, lignin and polysaccharide content. The inorganic content was quantified as ash.

Extractives were determined by successive Soxhlet extractions with pure solvents: dichloromethane, ethanol and water during 6 h, 16 h and 16 h, respectively as described before (Alves-Ferreira et al., 2017). The extractives solubilized by each solvent were determined by mass difference of the solid residue after drying at 105 ºC and reported as percent of the original sample (TAPPI 204 cm-97). The phenolic profile was obtained by Capillary Zone Electrophoresis (CZE), according to the method described by Moniz et al. (2018). Compounds were detected at 280 nm and analyzed by comparison of their UV spectra and migration times using authentic standards. Acid-insoluble (Klason) lignin and -soluble lignin were determined on the extracted samples by acid hydrolysis with 72% H_2SO_4: Klason lignin was determined as the mass of the solid residue after drying at 105 ºC (TAPPI 222 om-02) and the acid-soluble lignin was determined in the filtrate by UV spectroscopy at 206 nm (TAPPI Useful Method UM 250). Total lignin was defined as the sum of the Klason and acid-soluble lignins. Measurements were reported as a percentage of the original sample. The remaining acid solution was kept for sugar analysis

The polysaccharides were estimated by the content in neutral and acid monosaccharides (arabinose, xylose, galactose, mannose, glucose and galacturonic acids) as well as acetic acid in the hydrolysate obtained from the lignin determination. High performance ion chromatography with pulsed amperometric detection (HPIC-PAD) was used to quantify the monosaccharides in the hydrolysate of each sample, using a Dionex ICS-3000 system (Dionex, Sunnyvale, CA) equipped with a Carbopac PA10 (250 x 4 mm) plus Aminotrap columns. The separation was carried using a linear gradient of NaOH and CH_3COONa solutions as eluent at a flow rate of 1mL/min (0±20 min 18 mM NaOH; 20±25 min 50 mM NaOH+170 mM CH_3COONa; 25±40 min 50 mM NaOH+170 mM CH_3COONa); the column temperature was maintained at 30 ºC. The content of acetic acid was also determined in the hydrolysate using a High-Pressure Ion-exclusion

Chromatography with a UV/Visible detector (HIPCE-UV 210 nm) using a Thermo Finnigan Surveyor equipped with a BioRad Aminex 87H column (300 x 7.8 mm). The separation was achieved using a 10 mM H_2SO_4 mobile phase at a flow of 0.6 mL/min. The column temperature was 30 ºC.

The ash content was determined according to TAPPI 211 om-02, by incinerating 2.0 g of material at 525 °C overnight and the residues weighed and reported as mass percentage of the original samples.

2.4. Composition of ethanol-water extracts

Extracts were prepared using approximately 1 g sample and ethanol/water (50/50, v/v), with a 1:10 (m/v) solid-liquid ratio for 60 min at 50 ºC using an ultrasonic bath. After filtration, the solubilized extractives were determined by mass difference of the solid residue after drying at 105 ºC and reported as percent of the original sample. The supernatant extract was used for the quantitative analysis of total phenolics, flavonoids and condensed tannins, and for the determination of the antioxidant activity. Each assay was performed at least three times and at least three independent replicates were prepared for each standard and sample.

The total phenolic content of the extracts was determined by the Folin–Ciocalteu method (Singleton and Rossi, 1965). An aliquot (100 µL) of the extract was mixed with 4 mL of the Folin–Ciocalteu reagent and 4 mL of a 7% Na_2CO_3 solution. The mixture was kept for 5 min at 50 °C and, after cooling, the absorbance at 760 nm was measured. A calibration curve was built using gallic acid as a standard (0-150 µg/mL). The total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per g of extract.

Total flavonoids content was determined using the aluminum chloride colorimetric method (Zhishen et al., 1999). The calibration curve was prepared with catechin. An aliquot (1.0 mL) of the extract was mixed with 4.0 mL of deionized water and 0.3 mL of 5% $NaNO_2$ solution. After 5 min, 0.3 mL of a 10% $AlCl_3{\cdot}6H_2O$ solution was added to the mixture. After 5 min, 2.0 mL of 1 M NaOH solution were added, and absorbance at 510 nm was measured. The total flavonoid content was calculated as mg of (+)-catechin equivalents (CE)/g of the dry extract.

Tannins content was determined by the vanillin-H_2SO_4 method (Abdalla et al., 2014). The calibration curve was prepared with catechin. An aliquot (1.0 mL) of the extract was incubated for 15 min in the presence of 2.5 ml of 1.0% (m/v) vanillin and of 2.5 ml of 25% (v/v) sulphuric acid, both diluted in absolute methanol. The blank solution was

prepared without vanillin. The absorbances were measured at 500 nm and results were expressed as mg of catechin equivalents (CE)/ g of the dry extract.

2.5. Antioxidant activity

Two methods were used to determine the antioxidant properties of the samples: ferric reducing/antioxidant power (FRAP), which measures the sample's ferric reducing power, and 2,2-diphenyl-1-picryhydrazyl (DPPH), which measures the free radical scavenging capacity.

2.5.1. Ferric-reducing antioxidant power (FRAP)

The FRAP method is based on the reduction at low pH of a colorless ferric complex (Fe^{3+}- tripyridyltriazine) to a blue-colored ferrous complex (Fe^{2+}- tripyridyltriazine) by the action of electron-donating antioxidants (Benzie and Strain 1996). Briefly, 90 µL of the extracts was mixed with 270 µL of distilled water and 2.7 mL of freshly prepared FRAP reagent (25 mL of acetate buffer (0.3 M, pH 3.6), 2.5 mL of TPTZ (tripyridyl triazine) (10mM) diluted in HCl (40 mM) and 2.5 mL of $FeCl_3 \cdot 6H_2O$ (20 mM) was added. The absorbance at 595 nm was measured after 5 min. A standard curve was prepared using various concentrations of $FeSO_4 \times 7H_2O$. FRAP values were expressed in millimolar (Fe^{2+}/g of the sample).

2.5.2. DPPH radical scavenging assay

The DPPH assay was performed using 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) (Sharma and Bhat, 2009) and expressed in terms of: a) the amount of extract required to reduce 50% of the DPPH concentration (IC_{50}); and b) the Trolox equivalents on a dry extract base. Solutions with different concentrations were prepared of the initial extract and of Trolox solution in methanol (0.2 mg/mL). An aliquot of 100 µL of each methanolic solution of extract and Trolox was added to 3.9 mL of a DPPH methanolic solution (24 µg/mL). The blank sample consisted of 100 µL of methanol added to 3.9 mL of DPPH solution. The absorbance of the extracts was measured at 515 nm and compared to the initial absorbance of the DPPH solution using methanol as blank. The radical scavenging activity of each sample was calculated by the DPPH inhibition percentage as follows: $I\% = [(Abs_0-Abs_1)/Abs_0] \times 100$, where Abs_0 was the absorbance of

the blank and Abs_1 was the absorbance in the presence of the extract at different concentrations.

The IC_{50} inhibiting concentration, which represents the concentration of a sample necessary to sequester 50% of the DPPH radicals, was obtained by plotting the inhibition percentage against the extract concentration. The scavenging effect on the DPPH radical of the extract was also expressed as the Trolox equivalent antioxidant capacity (TEAC) calculated from the calibration curve with the Trolox solution concentrations and the percentage of scavenging effect on the DPPH radical.

2.6. Pyrolysis experiments

The extracted samples of stem and leaves were powdered in a Retsch MM200 mixer ball mill. The samples (ca. 100 µg) were pyrolyzed in a quartz boat at 550 ºC for 10 s using a 5150 CDS apparatus linked to an Agilent GC 7890B coupled to a mass detector system 5977B using electron impact mode (EI at 70 eV). A fused-silica capillary column ZB-1701 (60 m x 0.25 mm i.d. x 0.25 µm film thickness) was used. The gas chromatography conditions and oven program were described in Şen et al. (2018). The pyrolysis products were identified by comparison with Wiley, NIST2014 computer libraries and by literature and assigned as derived from lignin, from S (syringyl), G (guaiacyl) and H (*p*-hydroxyphenyl) lignin monomeric units, and from carbohydrates. The compounds were calculated using their peak area as a percentage of the total peak area. Total lignin, the lignin monomeric ratios (S/G ratio and H:G:S), as well as total carbohydrates were calculated according to the sum of peak areas of the corresponding compounds.

3. Results and discussion

3.1. Bark and wood anatomy

The stem bark and wood anatomy of *C. ladanifer* is characterized in Fig. 1A-D and Fig.2 A-D). It shows similarities with other genus of the Cistaceae and is in accordance with the descriptions made for this species (Schweingruber et al., 2011, Crivellaro & Schweingruber, 2013).

Microscopy observations on the transverse section of the 3-4 year–old stem of *C. ladanifer* clearly distinguished three parts: pith, wood and bark (Fig. 1A-D).

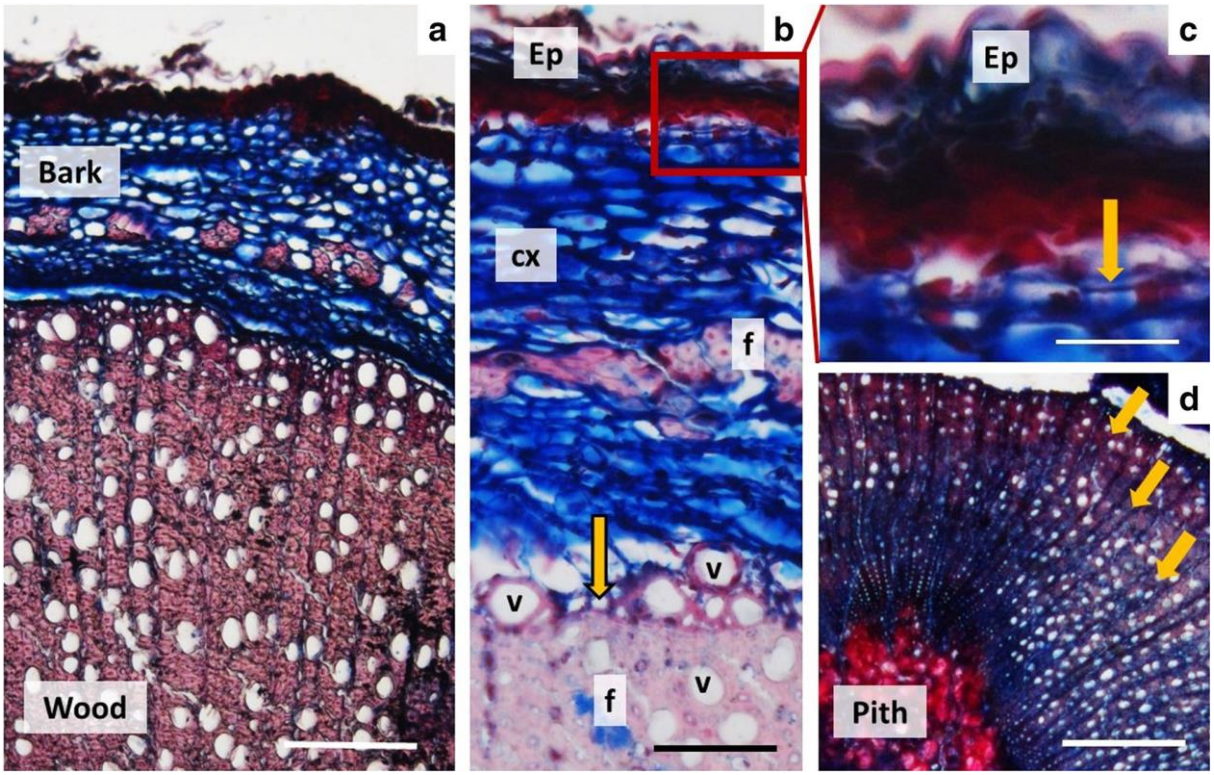

Fig. 1 General structure of a 3-year-old stem of *Cistus ladanifer* in transverse section. A: bark and wood; B: epidermis (Ep), cortex (cx) thickened walled fibers (f); vessels (v) scanty marginal parenchyma (arrow); C: Epidermis with cuticle and cell division of the phellogen (arrow); D: pith and wood annual rings (arrows). Scale bar: A=125 µm; B=50 µm; C=100 µm D= 250 µm

Bark (cortex and phloem) and wood (xylem) represent all the tissues outside and inside the vascular cambium, respectively. At this age, the bark still includes primary tissues represented by the epidermis (Ep) with a cuticle, and the cortex with large or small thin layered parenchyma cells (cx) (Fig.1A-B). The tangential divisions of cortical cells give rise to the phellogen that produces phellem to the outside and phelloderm to the inside, which together constitute the periderm (Figure 1C); the periderm is a protective tissue that replaces the epidermis when further growth in diameter takes place. The phloem includes highly thickened fibers in small groups or in tangential rows (mechanical tissue, f) and slightly dilated ray cells (storage and radial conducting tissue, r) (Fig. 1A); the sieve tube elements (conducting tissue) and parenchyma cells (storage tissue) are often difficult to differentiate in cross-section; numerous prismatic crystals are present as well as cell contents in parenchyma cells (Fig.2A).

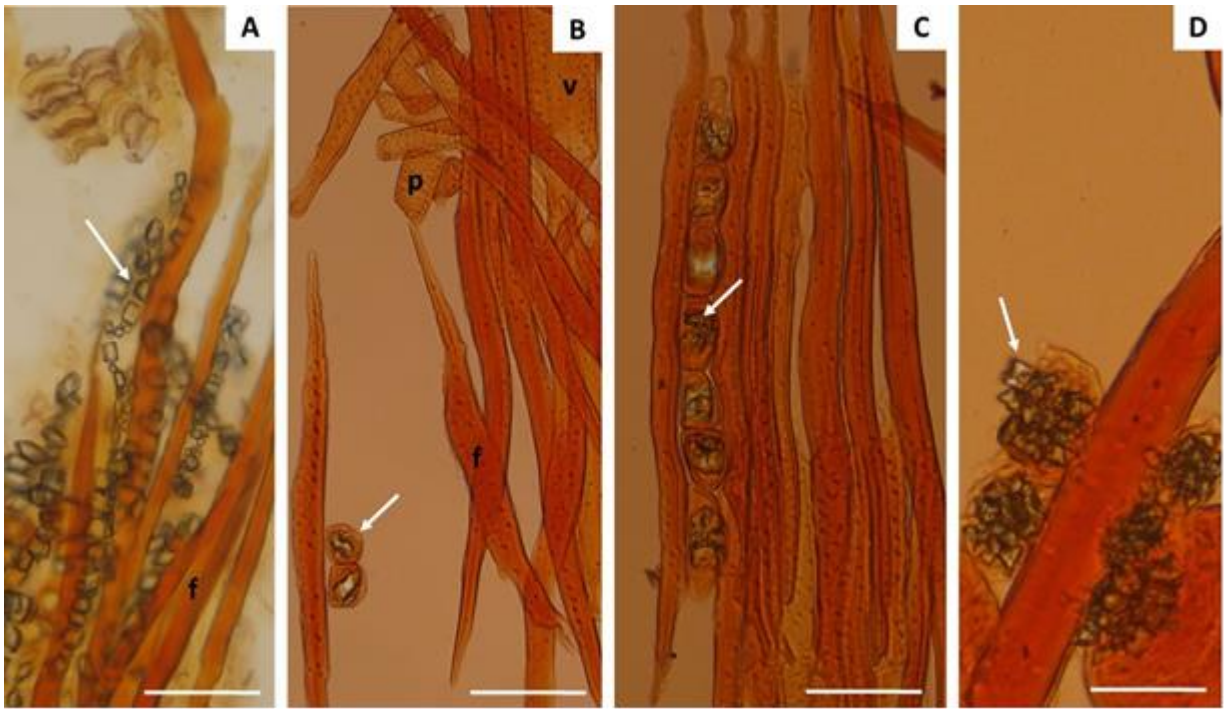

Fig. 2 Individualized cells of bark (A) and wood (B-D): fibres (f), parenchyma (p), vessels (v) and crystals (arrows). Scale bar: A-C = 50 µm; D= 100 µm

The xylem is characterized by the presence of growth rings (Fig. 1D, arrows) that are distinct by the presence of marginal parenchyma (Fig. 1B, arrow) and radially flattened and thick-walled fibers. The wood is semi-diffuse to diffuse porous, and the vessels are mostly solitary with alternate inter-vessel pits, thin helical thickenings and simple perforation (Fig.2B); the fibers are very thick walled and pitted (Fig.2B-D); the axial parenchyma is scarce, paratracheal and diffuse and the rays are thin. Prismatic crystals and druses are observed in the parenchyma cells (Fig. 2C-D)

The pith is round (Fig. 1D) with thick walled and content filled parenchyma cells. Accumulation of phenolics in various tissues in the xylem, pith and cortical parenchyma was also reported by De Micco & Arone (2007) in 1-year-old branches of *C. ladanifer,* suggesting a plant defense from animal predation and pathogens.

3.2. Chemical composition

The chemical composition of *C. ladanifer* biomass fractions (stems, branches, leaves and cysts) and of the residues obtained from two distilleries (CLR1 and CLR2) is given in Table 1.

Table 1. Summative chemical composition (% of total dry mass) of *Cistus ladanifer* biomass fractions (stems, branches, leaves and cysts) and of the residues (CLR1 and CLR2) obtained from two essential oil distilleries.

Chemical composition (% of total dry mass)	**Stems**	**Branches**	**Leaves**	**Cysts**	**CLR 1**	**CLR 2**
Ash	2.2	4.1	5.2	3.9	4.4	5.2
Total extractives	**10.8**	**31.9**	**53.7**	**33.5**	**43.7**	**39.2**
Dichloromethane	1.5	6.4	14.8	4.2	9.1	8.5
Ethanol	6.1	16.5	24.1	12.3	17.2	13.9
Water	3.3	9.0	14.9	17.1	17.5	16.9
Lignin total	**21.2**	**18.6**	**15.4**	**15.8**	**19.3**	**19.2**
Klason Lignin	18.2	16.8	13.6	14.0	17.6	17.6
Soluble Lignin	3.0	1.7	1.8	1.8	1.7	1.7
Polysaccharides	**59.5**	**40.4**	**23.3**	**42.4**	**29.2**	**33.3**

The different CL biomass fractions show distinguishable chemical differences. CL leaves are characterized by a very high extractives content (53.7%) with a significant proportion of lipophilic extractives (26% of total extractives), while stems have much lower extractives content (10.8%) mostly polar (soluble in ethanol and water), which represent 86% of the total extractives. The extractives in cysts and branches fractions were also high, 33.5 % and 31.9%, respectively and polar extractives were dominant, corresponding to 87.6% (cysts) and 80% (branches) of the total extractives.

The stem and branches showed similar cell wall lignification, respectively 21.2% and 18.6% of lignin, whereas in leaves and cysts the lignin content was about 15.5 %.

The monomeric composition of polysaccharides was similar for all biomass fractions (Table 2). The major monosaccharide was glucose (53.7% of the total monomeric units in stem and 42.8% in leaves) while xylans were the main hemicelluloses (xylose, arabinose and acetyl groups represented 36% and 42.9% of the total monomeric units, respectively in stem and leaves).

The chemical composition of the distillery residues (CLR) were on average: 4.8% ash, 41.5% total extractives, 19.3% lignin, 16.1% cellulose (with glucose representing 51.7% of total monosaccharides) and 15.2% hemicelluloses (xylose, arabinose and acetyl groups represent 33.7% of the total monosaccharides).

Galactose or mannose represented values from 1 % to 4 % of the total content of neutral sugars. Galacturonic acid was also detected in all the samples reaching a content from 2.4 % to 12.5 %.

Table 2. Monosaccharides and acid composition (% of total neutral monosaccharides) of *Cistus ladanifer* biomass fractions (stems, branches, leaves and cysts) and of the residues (CLR1 and CLR2) obtained from two essential oil distilleries.

Monosaccharides (% of units)	Stems	Branches	Leaves	Cysts	CLR 1	CLR 2
Arabinose	1.6	4.6	8.1	2.8	5.1	3.8
Xylose	31.9	28.9	22.3	30.7	22.7	27.2
Mannose	3.8	3.7	4.0	2.2	3.7	3.7
Galactose	1.2	2.9	6.7	1.9	4.0	3.2
Glucose	53.7	50.0	42.9	52.5	52.2	51.2
Galacturonic acid	2.4	5.0	12.5	3.5	8.5	6.1
Acetic acid	5.5	5.0	3.6	6.3	3.9	4.9

3.3. Phenolic composition of ethanol-water extracts

The extraction yields of the ethanol/water extracts of the *C. ladanifer* biomass fractions and CLR residues and their total phenolic, flavonoid and condensed tannin contents are shown in Table 3. The extraction yields depended on the material: the highest yield was in leaves (37.8%) followed by cysts and branches (21.1 and 20.6%) and was lowest in stems (5.8%). These yields are slightly lower than the sum of the Soxhlet extraction yields with ethanol (e.g. 6.1% in stem and 24.1% in leaves) and water (3.3% in stem and 14.9% in leaves) and are associated with the extraction conditions used, including the temperature, time of extraction and solid/liquid ratio. The yields of polar extracts of the CLR residues were 24.1 and 25.7%.

The composition of the extracts also differed among the various biomass fractions of *C. ladanifer*. Table 3 compares the composition of the different extracts with results expressed in milligrams per gram extract and in milligrams per gram biomass (starting material).

The stem extract showed the highest polyphenol content (420.9 mg GAE /g extract), leaf and cyst extracts displayed similar polyphenolic content (303.9-378.8 mg GAE /g extract) and that of the branches extract was lowest (287.1 mg GAE/g extract). When expressed on g GAE/100 g biomass, which may be of practical interest if this valorization route is envisaged, the difference between biomass components becomes more explicit because of the corresponding extraction yield values. Leaves had the

highest content of total phenolics (14.3 g GAE/100 g biomass), followed by cysts (6.4 g GAE/100 g biomass), branches (5.9 g GAE/100 g biomass), and stems (2.4 g GAE/100 g biomass).

Table 3. Extraction yield in ethanol/water (50:50) and antioxidant activities of *Cistus ladanifer* biomass fractions (stems, branches, leaves and cysts) and of the residues (CLR1 and CLR2) obtained from two essential oil distilleries.

	Stems	**Branches**	**Leaves**	**Cysts**	**CLR 1**	**CLR 2**
Extraction yield (%)	5.8	20.6	37.8	21.1	25.7	24.5
Total phenolic (mg GAE/g extract)	420.9	287.1	378.8	303.9	285.5	270.8
Total phenolic (mg GAE/g biomass)	24.3	59.0	143.1	64.1	73.5	66.4
Tannins (mg CE/g extract)	118.4	34.4	39.5	21.9	22.7	25.9
Tannins (mg CE/g biomass)	6.9	7.1	14.9	4.6	5.9	6.4
Flavonoids (mg CE/g extract)	86.8	57.4	41.8	32.3	33.3	39.0
Flavonoids (mg CE/g biomass)	5.0	11.8	15.8	6.8	8.6	9.6
Antioxidant capacity TEAC (mg Trolox/g biomass)	62.1	159.3	321.5	167.3	159.3	129.7
IC_{50} values (µg extract/mL)	3.2	4.6	4.0	4.7	5.0	6.3
FRAP (mM Fe^{2+}/g extract)	10.13	6.6	8.2	6.6	5.3	5.9
FRAP (mM Fe^{2+}/g biomass)	0.6	1.4	3.1	1.4	1.4	1.5

GAE: Gallic acid equivalents; CE: catechin equivalents

Comparable phenolic content was reported for ethanol-water extracts of *C. ladanifer* leaves (40.5 mg ferulic acid equivalents/g dry matter) and stem (36.9 mg ferulic acid equivalents /g dry matter) (Zidane et al., 2013) and for ethanolic leaves extracts of *C. salviifolius* and *C. monspeliensis* (49.9 and 56.4 mg GAE/g dry matter, respectively) and for water leaves extracts (54.6 and 37.4 mg GAE/g dry matter respectively) (Mahmoudi et al., 2016).

Regarding flavonoid content (Table 3), the extract of *C. ladanifer* stem was richer (86.8 mg CE/g extract) compared to leaves, cysts and branches (39.5, 32.3 and 34.4 mg CE/g extract respectively). Higher values were reported for ethanolic and methanolic extracts of *C. ladanifer* leaves (between 61.4 and 64.3 mg rutin/g extract) (Amensour et al., 2010). When expressed on g CE/100 g of starting material, the content of flavonoids ranged from 1.6 g CE/100 g dry leaves to 0.5 g CE/100 g of dry stem. Zidane et al. (2013) referred for ethanol-water extract of *C. ladanifer* leaves and stem, respectively 26.0 and 7.9 mg quercetin equivalents/g dry weight. The flavonoid contents in the ethanolic leaves extracts of *C. salviifolius* was reported as 27.8 g CE/100 g dry matter (Rebaya et al., 2016) and as 7. 0 and 5.3 mg CE/g dry matter, respectively, for ethanolic and water leaves extracts of *C. salviifolius* and *C. monspeliensis* (Mahmoudi et al., 2016).

The proportion of flavonoids in the total phenolics was low among all extracts, e.g. the flavonoids represented only about 20% of the total polyphenol content in stem and branches, and 11% in leaves and cysts.

Total phenolics and flavonoid contents are variable in *Cistus* and depend on the plant tissue evaluated, season, age and analytical methods (Andrade et al., 2009; Guimarães et al., 2010; Sánchez-Vioque et al., 2013; Guerreiro et al., 2016)

Condensed tannins were present in the extracts of all the *C. ladanifer* biomass fractions in variable amounts from 21.9 mg to 118.4 mg CE/g extract respectively for cysts and stems. When expressed in CE /100 g starting material, the leaves had the largest tannin content (1.5 CE/100 g starting material), and the stem the smallest value (0.7 g CE/100 g starting material), which is in tune with the corresponding extraction yields. Condensed tannin content for ethanol and water leaves extracts of *C. salviifolius* and *C. monspeliensis* were, respectively, 14.5 and 17.9 mg CE/g dry matter and 16.8 and 22.2 mg CE/g dry matter (Mahmoudi et al., 2016).

The ethanol-water extraction yield and composition of CLR residues are given in Table 3. The extract yield was on average 25.1 %, in close agreement with the chemical composition data (Table 2). The total phenolic content (278.2 mg GAE/g extract or 7.4 g GAE/100 g dry matter) was in the range of previously published values for whole plant extracts of *C. ladanifer.* Andrade et al. (2009) referred 255.2 mg GAE/g extract for ethanol extract of *C. ladanifer.* Barrajón-Catalán et al. (2010) referred 22.9 g GAE/100 g dry weight for *C. ladanifer* aqueous extracts. Tomás-Menor et al. (2013) found 13.3 g GAE/100 g dry weight in hydroalcoholic extract of the whole plant of *C. ladanifer*. Nicoletti et al. (2015) reported values for plants of different *Cistus* species: 40.5 mg GAE/g dry plant for *C. libanotis*, 32.5 mg GAE/g dry plant for *C. villosus* and 33.2 mg GAE/g dry plant for *C. monspeliensis*. Tomás-Menor et al. (2013) referred for *C. albidus* 21.8 g GAE/100 g

dry weight, for *C. clusii* 23.1 g GAE/100 g dry weight and for *C. salviifolius* 27.3 g GAE /100 g dry weight.

Total flavonoid and tannin contents of CLR residues extracts were 36.2 mg CE/g extract (0.9 g CE/100 g dry matter) and 24.3 mg CE/g extract (0.6 g CE/100 g dry matter). Barrajón-Catalán et al. (2010) referred 3.0 mg QE/100 mg dry weight for *C. ladanifer* aqueous extracts, and Andrade et al. (2009) referred 20.5 QE/g extract and 23.4 QE/g extract, respectively in ethanol and acetone extract. Tomás-Menor et al. (2013) reported for *C. ladanifer* 0.9 g QE/100 g dry weight, for *C. albidus* 1.1 g QE/100 g dry weight, for *C. clusii* 0.7 g QE/100 g dry weight and for *C. salviifolius* 1.6 g QE /100 g dry weight.

3.4. Antioxidant activity

The results for the antioxidant potential of the ethanol-water extracts of the *C. ladanifer* fractions using DPPH and FRAP assays are summarized in Table 3.

The DPPH radical scavenging activity is reported in terms of IC_{50} as well as of the Trolox equivalents (TEAC) on a starting material basis (mg TEAC g^{-1} of starting material). These extracts revealed high efficiency as free radical scavenger, with an IC_{50} value between 3.2 and 4.7 µg/mL when compared to Trolox (IC_{50} of 2.7 µg/mL). The IC_{50} of the CLR extracts also indicates high antioxidant activity (IC_{50} of 6.3 and 5.0 µg/mL), comparing very favorably with the IC_{50} values of well-known antioxidant standards such as catechin (5.4 µg/mL) and Trolox (2.7 µg/mL), the latter considered to have excellent antioxidant activity.

Substantially low values of IC_{50} were reported for aqueous extracts of *C. salviifolius* leaves (1.3-6.5 µg/mL) and *C. monspeliensis* leaves (1.2 µg/mL) (Mahmoudi et al., 2016; El Euch et al., 2015). Zidane et al. (2013) reported also high scavenging ability of DPPH radicals for methanolic extracts of different plant parts of *C. ladanifer* and *C. libanotis*.

All the extracts showed also strong ferric ion reducing activities that ranged from 6.6 to 10.1 mM Fe^{2+}/g extract (respectively for branches and stems). Expressed on the starting material, the leaves extract showed the highest ferric ion reducing capacities (308.2 mM Fe^{2+}/100 g) and stem extracts the lowest (58.6 mM Fe^{2+}/100 g). The CLR extracts also have strong ferric ion reducing capacities (5.9 and 5.3 mM Fe^{2+}/g extract, respectively, 145 and 135 mM Fe^{2+}/100 g). The results obtained for *C. ladanifer* are comparable to those previously reported as 117.7 mmol Fe^{2+}/100 g dry weight and 179.1 mmol Fe^{2+}/100 g dry weight for *C. populifolius* (Barrajón-Catalán et al., 2010) and 318.8

mmol Fe^{2+}/100 g dry weight for *C. salviifolius* aqueous extracts (Tomás-Menor et al., 2013).

3.5. Lignin composition

Figure 3 shows the pyrograms obtained for the stem and leaves of *C. ladanifer* and Table 4 lists the lignin-derived compounds obtained by pyrolysis, including CLR1 sample previously described (Alves-Ferreira et al., 2019) classified as derived from the H, G and S lignin units. From the total of 82 pyrolysis products that were identified in the pyrograms (data not shown), 29, 28 and 27 peaks were identified as originating from lignin-derived compounds for the stem leaves and CLR1, respectively.

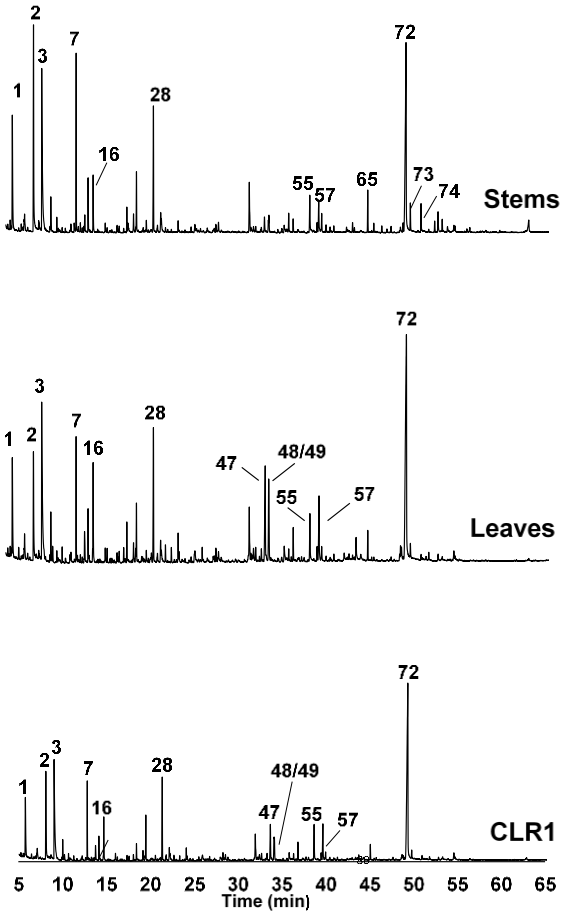

Fig. 3. Py-GC/MS chromatograms of steams leaves and residues of *C. ladanifer.* Main peaks: (1) 2-oxo-propanal, (2) hydroxyacetaldehyde, (3) acetic acid (7) 3-hydroxypropanal (16) furfural, (28) 4-hydroxy-5,6-dihydro-2H-pyran-2-one, (47) 1,5-anhydro-arabinofuranose, (48) 2,3-dihydrobenzofuran, (49) 4-vinylguaiacol, (55) 2-hydroxymethyl-5-hydroxy-2,3-dihydro-4*H*-pyran-4-one, (57) similar to 1,5-Anhydro-arabinofuranose, (65) 4-vinylsyringol, (72) 1,6-anhydro-β-D-glucopyranose, (73) *trans* 4-propenylsyringol, (74) syringaldehyde

The pyrograms (Fig. 3) show that the main lignin-derived compounds obtained from pyrolysis of the stem were 4-vinylsyringol (peak 65, S), *trans* 4-propenylsyringol (peak 73, S) and syringaldehyde (peak 74, S) while those from leaves were 2,3-dihydrobenzofuran (H) and 4-vinylguaiacol (G) (peak 48/49 overlapped). There was a clear difference in the lignin composition of stem and leaves: the H:G:S ratio was 1:25:50 for stem lignin and 1:1.4:1 for leaves lignin (Table 4). Stem lignin is an SG-lignin composed predominantly of S-units (65.8% of units) with only very minor amount of H units, whereas lignin in leaves is an HSG lignin constituted mainly by G-units (41.2% of units) but with substantial and equal amounts of S and H units (29.4%). CLR presented a S/G ratio similar to leaves (0.9 *vs* 0.7), evidencing once again the strong presence of leaves in these type of residues (Table 4).

Table 4. Identification of the lignin-derived pyrolysis products (as % of the pyrogram peak areas) in stems and leaves of *Cistus ladanifer*

Lignin products	**Retention Time**	**% of the total area**		
		Stems	Leaves	CLR1[a]
Siryngol derivatives (S)				
syringol	36.38	0.8	0.4	0.4
4-methylsyringol	40.02	0.8	0.3	0.6
4-ethylsyringol	42.89	0.1	0.2	0.1
4-vinylsyringol	45.11	1.5	0.8	0.8
4-allylsyringol	45.78	0.3	0.1	0.1
cis 4-propenylsyringol	47.68	0.2	0.1	0.2
4-propinylsyringol	49.02	0.3	0.1	0.1
trans 4-propenylsyringol	49.82	1.1	0.5	0.4
syringaldehyde	51.01	1	0.1	0.2
homosyringaldehyde	52.57	0.4	n.d.	n.d
acetosyringone	53.34	0.5	0.1	0.2
syringylacetone	54.74	0.2	0.2	0.2
trans sinapaldehyde	62.90	1	0.1	0.2
Guaiacol derivatives (G)				
guaiacol	24.16#	0.2	0.6	0.3
4-methylguaiacol	28.32	0.4	0.4	0.6
4-ethylguaiacol	31.82	0.04	n.d.	0.05

4-vinylguaiacol	34.15#	0.7	1.6	1.2
eugenol	35.16	0.1	0.1	0.1
trans isoeugenol	39.50	0.4	0.5	0.5
vanillin	40.50	0.3	0.2	0.2
1-(4-hydroxy-3-methoxyphenyl)-propyne	40.95	0.2	0.1	0.1
1-(4-hydroxy-3-methoxyphenyl)-propyne	41.36	0.3	0.2	0.2
homovanillin	42.76	0.2	n.d.	0.1
acetoguaiacone	43.64	0.2	0.2	0.1
guaiacylacetone	45.55	0.1	0.1	0.1
trans coniferyl alcohol	47.22#	0.1	n.d.	n.d
guaiacyl vinyl ketone	47.22#	0.1	n.d.	n.d
trans coniferyl alcohol	53.93	0.1	n.d.	n.d
trans coniferaldehyde	54.65	0.3	0.2	0.6
***p*-Phenylphenol derivatives (H)**				
phenol	23.38	0.1	0.4	0.2
o-cresol	25.33	n.d.	0.4	n.d
p-cresol	26.79	n.d.	0.4	0.2
m-cresol	26.87	n.d.	0.1	n.d
2,3-dihydrobenzofuran	34.15#	n.d.	1.6	n.d
2,3-dimethyl-phenol	28.72	n.d.	0.1	n.d
Not determined lignin source (NDL)				
toluene	10.26	n.d.	0.4	0.3
styrene	14.29	n.d.	0.2	0.1
S		8.0	3.1	3.5
G		3.7	4.3	4.0
H		0.1	2.9	0.5
S/G		2.2	0.7	0.9
H:G:S		1:25:50	1:1.4:1	1:1.1:0.1
NDL		n.d.	0.7	0.4

- compounds overlapped; n.d. - not detected; [a] Some values are published in Alves-Ferreira et al. (2019

It has been shown that lignin composition varies between species and between tissues in a plant (Lourenço and Pereira, 2018, 2016). Micco & Aronne (2007) found a predominance of S units in xylem and of G units in the pith of 1-year-old branches of *C. ladanifer*. Lignin composition also differs between the different morphological parts of the banana plant e.g. leaf sheaths (H:G:S of 1:2:0.5), leaf blades (1:9.3:6.3) and floral stalks (1:1.6:1) (Oliveira et al. 2007). The study of lignin in xylem, phloem and cork of *Quercus suber* showed that the different tissues presented different H:G:S compositions: lignin from xylem is enriched in S-units (1:45:55), the lignin from phloem has less S and more G-units (1:58:41) and the cork lignin is enriched in G-units (2:85:13) (Lourenço et al., 2016).

The S/G ratio of lignin in *C. ladanifer* stem and leaves was 2.2 and 0.7, respectively. The S/G ratio is an important parameter for the chemical evaluation of lignin especially regarding pulping potential due to the higher reactivity of the S-units (Lourenço and Pereira, 2018). Thus, the stem of *Cistus* biomass is more suitable for delignification processes, while the leaves may not be very appropriate for this type of chemical approach. Although the industrial valorization of lignins is still rare, their conversion into chemicals will positively influence the economic viability of lignocellulosic biorefineries (Wild et al., 2012).

3.6. Phenolic profile of Soxhlet extracts

The phenolic profiles obtained by CZE at 280 nm for the ethanolic and water Soxhlet extractives are shown in Fig. 4 and 5.

From the electropherograms of the extractives solubilized in ethanol shown in Figs 4 and 5, it can be seen that the phenolic profiles are highly complex and in agreement with the results obtained for the total phenolics content. In Fig. 4A-F, it is possible to observe the presence of the flavonoid apigenin (peak 1) in the samples of cysts, leaves and CLR2. A band showing the presence of gallocatechins (peak 3) and gallic acid (peak 6) confirm the presence of tannins and were the main polyphenols in the stem extractives. In fact, these compounds were also detected in the leaves and branches extractives. In addition, gallic acid was also found in CLR1 samples. Branches extract was the only sample where the presence of isoquercetin (peak 2) was detected. Peaks 4 and 5 in the samples of cysts, leaves and branches, could not be identified. Other compounds such as hydroxybenzoic acid and vanillic acid were also found in the ethanolic extractives at 200 nm (data not shown).

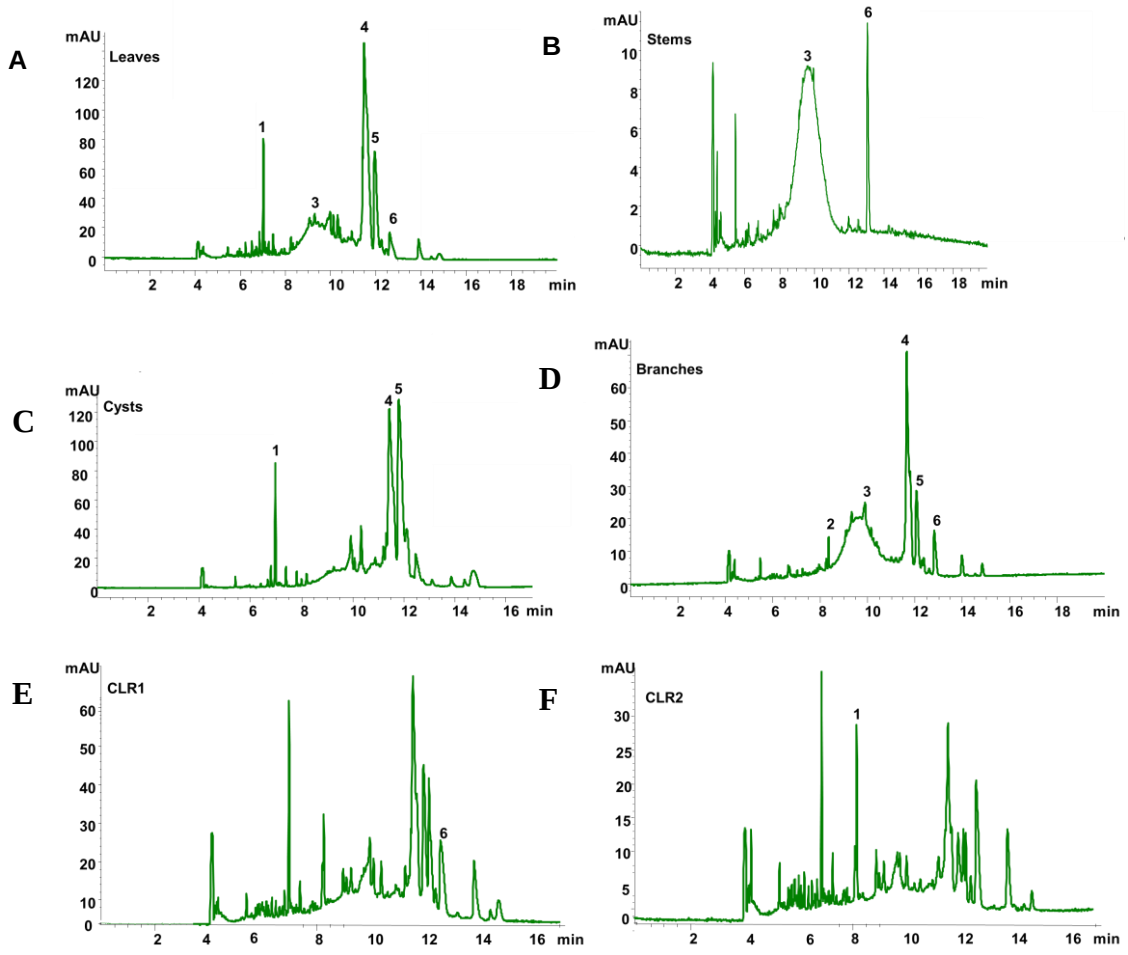

Fig. 4 Electropherograms (280 nm) showing the phenolic profile of ethanolic extracts of *Cistus* fractions (leaves, stems, cysts, branches and distillery residues (CLR1, CLR2). Peak identification: (1) apigenin, (2) isoquercetin, (3) gallocatechins, (4) unidentified compound, (5) unidentified compound, (6) gallic acid. See text for CZE for phenolics separation.

For the water extractives, the phenolic profile also shows some complexity, but only gallic acid could be identified with the available standards. As an example, the electropherograms obtained for leaves and CLR1 can be seen in Fig. 5. Gallic acid, gallocatechin, rutin, apigenin and vanillic acid from cistus extracts were already determined in previous studies (Chaves et al., 1998; Barrajón-Catalán et al., 2010; Fernández-Arroyo et al., 2010; Barros et al., 2013; Tomás-Menor et al., 2013; Alves-Ferreira et al. 2019). In fact, *C. ladanifer* extracts were characterized in several works, since this plant is traditionally recognized by its medicinal properties. The studies on the phenolic composition of *C. ladanifer* extracts indicate mainly the presence of gallotannins, flavonoids and phenolic acids. However, ellagic acid derivatives were the most abundant

group determined by Barros et al. (2013) in aqueous methanolic extracts of CL, with punicalagin gallates as the main compounds. Tomás-Menor et al. (2013) reported differences between aqueous and hydroalcoholic extracts of *C. ladanifer*, with this latter presenting flavonoid derivatives such as apigenin methylether and kaempferol dimethylether. Other compounds such as quercetins, kaempferol derivatives, 49-methyl-apigenin, 7-methyl-apigenin, quinic acid, 3-β-D-glucoside and cornusiin B ellagitannins as punicalin, punicalagin and gallagic acid were previously reported in different extracts of cistus (Chaves et al., 1998; Fernández-Arroyo et al., 2010; Barros et al., 2013; Tomás-Menor et al., 2013).

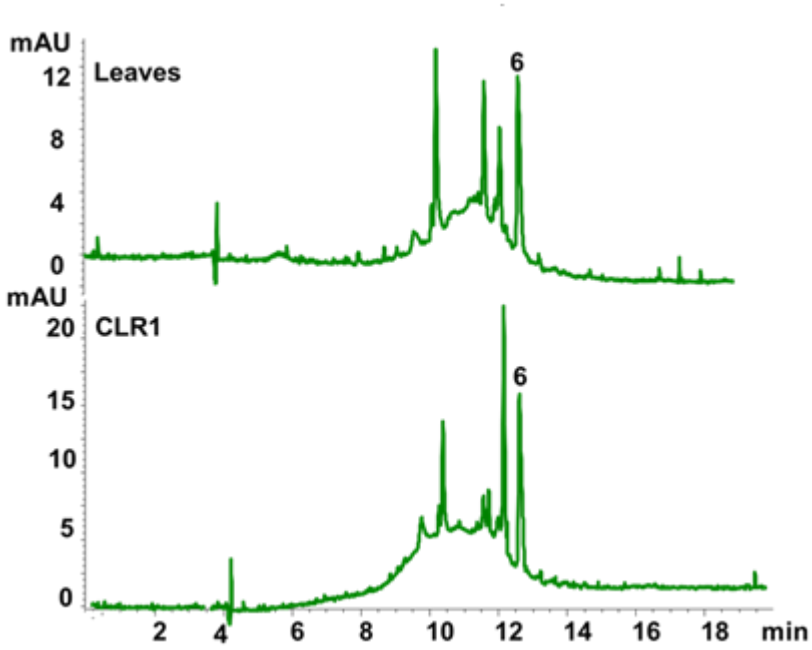

Fig. 5 Electropherograms (280 nm) showing the phenolic profile of the aqueous extracts of *Cistus* fractions exemplified by two samples (leaves and distillery residues (CLR1)). Peak identification: (6) gallic acid. See text for CZE for phenolics separation.

Various studies were also conducted in order to know the profile of the volatile components present in the lipophilic extracts of *C. ladanifer*. For dichloromethane extracts from leaves of CL, 2,2,6-trimethylcyclohexanone, acetophenone and 2-phenylethanol were the main compounds detected, being these molecules extremely aromatic (Ramalho et al., 1999). Other volatiles as α-pinene, camphene, camphor, fenchone and verbenone from shoots of CL extracted with hexane were also identified (Santos et al., 2016). Morales-Soto et al. (2015) described the volatile profile of *Cistus* plants and determined

51 compounds for samples of *C. ladanifer*, with monoterpenes and sesquiterpenes as the main families of lipophilic compounds.

Thus, the results of this work showed that a high quantity of different constituents was identified from Soxhlet ethanolic extracts, but also aqueous extracts revealed to have a complex matrix, although only gallic acid could be identified by CZE.

4. Conclusions

The biomass components of the *C. ladanifer* plants e.g. stem, branches, leaves and cysts show different chemical composition, mostly regarding the content of extractives which are particularly high in leaves e.g. fivefold the content in stems, while lignin content is highest in stems and branches. Lignin composition also show differences with more S-units in stem lignin and important amounts of H-units in leaves lignin. The distillery residues (CLR) have a composition resulting from the combination of the different biomass fractions. An important chemical feature of *C. ladanifer* components and of their distillery residues (CLR) is the high content of extractives, especially of polar extractives that were rich in phenolics, namely flavonoids and tannins, which showed high anti-oxidant activity.

The high extractive contents of *C. ladanifer* point out to the potential use of this species as a source of phytochemicals by applying extraction procedures. The remaining lignocellulosic material after extraction may be directed towards potential lignin and carbohydrates applications.

The use of *C. ladanifer* biomass for an extractives-lignocellulosic-based biorefinery therefore represents a potential valorization that may contribute to an additional revenue for the present essential oil distilleries.

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CAPÍTULO IV

Hydrothermal treatments of *Cistus ladanifer* industrial residues obtained from essential oil distilleries

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Abstract

Purpose The aim of this study was to optimize hydrothermal treatments (autohydrolysis) for selective hydrolysis of hemicelluloses in the residues obtained from the industrial steam distillation of *Cistus ladanifer* (rock-rose) for essential oil extraction (CLR). The effect of the autohydrolysis in the removal of extractives of these residues was also evaluated.

Methods The raw material was treated with water in a 6:1 liquid-to-solid ratio (w/w) and the effect of temperature (130–230ºC) on the composition of liquid and solid phases were evaluated and interpreted using the severity factor (log R_0).

Results The highest recovery of oligosaccharides (24.7 g/L) was obtained at moderate log R_0 of 3.12. Together with hemicelluloses, polar extracts were also largely solubilized during the hydrothermal process yielding hydrolysates with a relevant concentration of phenolics. The highest glucan content (35.6 g/100 g of CLR) of the solid residue was obtained for a high log R_0 of 4.0 (220 ºC). There was no apparent lignin solubilization in any of the conditions, which is an advantage of this hydrolytic pretreatment.

Conclusions Cistus ladanifer residues demonstrated to have potential to be used in the biorefinery framework with a full upgrade of all biomass fractions.

Keywords: Autohydrolysis, Biorefinery, *Cistus ladanifer* residues, Extractives, Oligosaccharides

1. Introduction

The management of forest understory biomass, including shrubs, is a relevant issue, given the potential environmental problem related to the risk of forest fire spread. Unfortunately, its management is not economic viable, as typically these species have no or low economic value. The development of effective valorization routes for these species can contribute to their industrial usage and hence promoting their sustainable environmental management. In this framework, the development of small-scale, and locally adapted biorefineries may be an effective approach to contribute to the development and strengthen of both the environmental security and local economy.

Cistus ladanifer (rock rose) is one example of such perennial shrub species native to the Mediterranean region and especially distributed in the Iberian Peninsula (Morales-Soto et al., 2015). In Spain (large mountainous regions), it is estimated that more than 10,000 km2 are covered by this species (Rincón et al., 2000) and in Portugal, it is abundant throughout the continental territory, especially in the southern and interior regions (Clamote et al., 2016). *C. ladanifer* produces a high-value essential oil that is generally obtained from the leaves and stems by hydrodistillation carried out commercially in small scale industrial units (Mariotti et al., 1997). This essential oil is particularly interesting for cosmetics and perfumes (Nuñez-Oliveira et al., 1995; Gomes et al., 2005; Andrade et al., 2009) and has been demonstrated to have antimicrobial and antioxidant potential (Greche et al., 2009; Zidane et al., 2013). Nevertheless, the essential oil content is rather diminute (typically below 0.5%) (Gomes et al., 2005). The remaining material can be used to produce an aromatic exudate or sticky resin known as labdanum, which has been used as a natural fixative in perfumery (Gomes et al., 2005), but its market is limited; typically, in Portugal, this residual material is used as fuel (firewood) for subsequent hydrodistillations after drying at ambient conditions (usually drying 1 day).

These solid residues obtained from *C. ladanifer* distilleries (CLR) are lignocellulosic materials and other alternatives are being proposed namely the production of biofuels or bioproducts. Indeed, the chemical composition of these residues, in particular, the polysaccharides and lignin contents (Ferreira et al., 2009; Gil et al., 2012; Ferro et al., 2015) make them adequate raw-materials for the valorization in a biorefinery framework.

In order to obtain different products, the fractionation of polymeric components of biomass (hemicelluloses, cellulose and lignin) is required e.g. by using pretreatment processes. Depending on the targeted fraction, different options can be followed including chemical, physicochemical and biological processes (Carvalheiro et al., 2011). Hydrothermal processes such as autohydrolysis have been employed in the treatment of

lignocellulosic biomass for partial or total solubilization of the hemicellulosic fraction allowing the production of hydrolysates containing oligomeric sugars, in particular xylo-oligosaccharides. Besides these compounds, monomeric sugars, and other components of biomass such as phenolic compounds and acetic acid are also obtained together with a minor production of sugar degradation compounds (e.g. furfural, 5-hydroxymethylfurfural and formic and levulinic acids). The pretreated solid residue obtained is enriched in cellulose and lignin (Garrote et al., 2002a; Rivas et al., 2002; Carvalheiro et al., 2004).

The aim of this work is to study the use of mild autohydrolysis processes to selectively hydrolyse CLR hemicelluloses, producing hemicellulose-derived oligosaccharides and to extract water-soluble phenolic compounds targeted to potential added-value applications (e.g. as bioactive products), as well as to obtain cellulose/lignin enriched solids, as a first step in the valorization of this residual biomass material in a biorefinery framework. This fractionation route will allow a new perspective for the valorization of *C. ladanifer* residues in a biorefinery framework, thereby contributing to an economic valorization of this natural resource.

2. Materials and methods

2.1. Raw material

Cistus ladanifer residues (CLR) used in this work consist of 2 to 4-year-old whole plants that were steam distilled to produce commercial essential oils in a small scale industrial unit. After processing, the CLR was dried at room temperature and ground with a knife mill to particles < 6 mm and stored in plastic containers at room temperature.

For characterization of the biomass partitioning, 2-3- and 5-year-old plants were collected from the field in February 2014 and May 2015. The whole plant was weighed and their organs were manually separated into leaves and stems of diameter between 4 mm and 2 cm (thin) and 2 and 10 cm (thick) and weighed.

2.2. Autohydrolysis

The hydrothermal treatments (autohydrolysis) were carried out in a 600 mL stainless steel reactor (Parr Instruments Co, USA). The raw material was mixed with water in a 6:1 liquid-to-solid ratio (w/w) and the agitation speed was set at 150 rpm. The reactor was heated to final temperatures ranging from 130 to 230 °C. Typically, the

average heating rate (from 100 °C) was 4.9 °C/min. When the desired temperature was attained, heating was stopped and the reactor was immediately cooled down by water passing the internal cooling coil and by placing the reactor in an ice bath (cooling time average until 100 °C below 3.0 min). This approach, using non-isothermal conditions has several advantages for understanding the overall process kinetics and better mimics the envisaged industrial process (Branco et al., 2015).

The liquid and solid phases were separated using a hydraulic press (Sotel, Portugal). The liquid phase was filtered (Whatman filter paper no. 1) and the pH was measured. The solid phase was extensively washed with water, dried at 50 °C and milled to particles smaller than 0.5 mm before analysis.

The reaction conditions were evaluated using the severity factor (log R_0), calculated by the equation proposed by Overend & Chornet for non-isothermal systems (Overend & Chornet, 1990):

$$R_0 = \int_0^t exp\left(\frac{T(t) - T_{ref}}{\omega}\right)dt$$

where *t* is time, *T* is temperature, *Tref* a reference temperature (100 °C) and ω is a constant that expresses the average influence of the temperature on the reaction. The standard empirical value of 14.75 proposed by Overend & Chornet was used (Overend & Chornet, 1987).

2.3. Analytical assays

The raw material (CLR) and the processed solids (after autohydrolysis) were Soxhlet extracted with three successive extractions with dichloromethane (6 h), ethanol (16 h) and water (16 h). The extractive-free solids were subjected to quantitative acid hydrolysis (QAH) with 72% (w/w) H_2SO_4 followed by hydrolysis with 4% (w/w) H_2SO_4, at 121 °C for 1 h. The solid residue after hydrolysis was corrected for ash and considered as Klason lignin. The hydrolysates resulting from QAH were filtered through 0.45 μm membranes and analyzed for monosaccharides (glucose, xylose, and arabinose) and acetic acid by HPLC (Agilent, Germany) equipped with RI and UV detectors and an Aminex HPX-87H column (Bio-Rad, USA) under previously described conditions (Moniz et al., 2013). In the case of the raw material, both extracted and non-extracted ("as it is") samples were analyzed.

The moisture content was determined by oven-drying at 100 °C to constant weight and the ash content was determined at 550 °C using NREL/TP-510-42622 protocol

(Sluiter et al., 2008). The determination of protein was carried out according to the Kjeldahl method (AOAC, 1975).

The liquid phase was directly analyzed by HPLC or after a post-hydrolysis, with 4% (w/w) H_2SO_4, at 120 °C for 60 min in an autoclave to hydrolyze oligosaccharides to their monomeric sugars. Sugars, acetic acid and degradation products (furfural, HMF, formic acid) were analyzed using the same column described above as detailed elsewhere (Moniz et al., 2013). Total phenolic compounds were determined by the Folin–Ciocalteu colorimetric method using a microplate spectrophotometer (Thermo Scientific, USA) adapted by Roseiro et al. (2013). Total phenolic compounds are expressed as mg of gallic acid equivalents (GAE)/mL).

3. Results and discussion

3.1. Raw material characterization

Table 1 shows the mass partitioning of *C. ladanifer* plants with different ages. The results indicate an age-dependent biomass component distribution with a higher proportion of leaves in younger plants and an increasing contribution of thick and thin stems with age. Total weight of the plants increased with age: for instance, 3-year-old plants weighed 4.7 times more than 2-year-old plants (1.324 *vs*. 0.279 kg), whereas 5-year-old plants weighed about two times more than the 3-year-old plants (2.977 *vs*. 1.324 kg).

Table 1. Biomass components (kg fresh weight)) of the aerial part of *Cistus ladanifer* plants of different age

Plant parts	**Biomass (fresh kg)**		
	2-years	***3-years***	***5-years***
Leaves	0.095±0.01	0.342±0.20	0.638±0.49
Stems, thin diameter	0.080±0.01	0.549±0.24	1.339±0.73
Stems, thick diameter	0.099±0.01	0.416±0.20	0.984±0.49
Flowers	-	-	-
Cysts	0.011±0.0	0.036±0.01	0.034±0.02
Total weight	0.279±0.02	1.324±0.62	2.977±1.7

The chemical composition of CL whole plants or residues found in the literature together with the data obtained in this work for the CLR is shown in Table 2. In this work,

the contents of glucan and hemicelluloses (estimated from xylan, arabinan and acetyl groups) accounted respectively for 16.1 and 13.2% for the initial samples (CLR), and for 27.8 and 22.7% for the extractive-free samples (CLRext). Glucan values are lower and hemicellulose content is higher than those reported by Ferro et al. (2015) for 10-year-old plants.

Table 2. Chemical compositions (% of dry weight) of *Cistus ladanifer* distillery residues determined as received (CLR) and after removal of extractives (CLRext) and comparison with literature data

Component	Whole plant[c] (Ferro et al., 2015)	Whole plant[b,c] (Paulino, 2013)	Residues (Gil et al., 2013)	Distillery residues (CLR)[a] This work	Distillery residues (CLRext)[b] This work
Cellulose	34.9±4.0	22.9±0.1	-	16.1±0.1[d]	27.8±0.1[d]
Hemicelluloses	6.6±3.8	22.0±0.1	-	13.2±0.6	22.7±0.9
Xylan	-	14.7±0.1	-	8.0±0.3	13.8±0.5
Arabinan	-	nd	-	2.5±0.3	4.3±0.5
Acetyl groups	-	7.3±0.08	-	2.7±0.1	4.61±0.1
Klason Lignin	15.6±0.2	26.7±0.7	30.4±0.2	17.0±1.0	29.5±1.8
Soluble Lignin				1.8±0.1	3.0±0.1
Ash	3.1±0.1		8.3±1.8	4.3±0.1	4.0±0.1
Protein	9.2±0.	1.50±0.6	-	5.7 ± 0.30	-
Extractives	6.2±0.8[e]	-	7.4±0.3[f]	42.2±1.5[g]	-

[a] *Non-extracted ("as it is" sample)*; [b] *extractive free sample;* [c] *10-year-old;* [d] *as glucan;* [e] *Extractable in acetone;* [f] *Extractable in ethanol and water,* [g] *Extractable in dichloromethane, ethanol and water*

Regarding extractives, the results obtained in this work for CLR showed a very high content, considerably above the values obtained by the other authors. The important percentage of leaves (Table 1), contributes to this high amount of extractives. The polar extractives (solubilized in water and ethanol) were dominant over the nonpolar extractives (solubilized in dichloromethane), corresponding to 79.8% of the total extractives. In agreement with the literature, CLR also contains significant amounts of lignin. Protein content of CLR was higher than the reported by Paulino (2013) but lower than that obtained by Ferro et al. (2015). The ash content values are about 50% lower than those reported by Gil et al. (2012) and about 38% higher than those determined by Ferro et al. (2015). It should be noted that according to the literature (Gil et al., 2012; Paulino, 2013; Ferro et al., 2015), *C. ladanifer* presents a very diverse composition. The following ranges

are reported: 16–35% for cellulose content, 7–23% for hemicelluloses content and 16–33% for lignin content. The amount of extractives also varies a lot although it largely depends on the extraction procedure. The present results and the values reported in the literature (Tables 1, 2) show that variability should be expected when considering *C. ladanifer* as a feedstock with seasonability and age of plants also playing a role. Therefore, valorization processes design must take into account the variation in amount, component distribution and chemical values.

3.2. Composition of the liquid phase

Table 3 shows the composition of the liquor obtained after autohydrolysis as a function of the severity factor (log R_0 ranging from 1.70 to 4.29). The results showed that for the lower severity conditions most of the sugars produced are in the oligomeric form.

Table 3. Composition of liquid fractions obtained after autohydrolysis of *Cistus ladanifer* distillery residues under different severity factors

(g/l)	**Severity factor, log *Ro***									
	1.70 (130 °C)	1.97 (150 °C)	2.57 (170 °C)	2.86 (180 °C)	3.12 (190 °C)	3.42 (200 °C)	3.57 (205 °C)	3.70 (210 °C)	4.00 (220 °C)	4.29 (230 °C)
GlcOS	4.6	7.1	9.0	8.3	9.3	8.3	8.1	8.0	4.4	1.9
XOS	0.0	0.3	1.9	3.9	6.9	9.6	11.0	10.1	3.8	0.0
AOS	2.0	6.0	8.7	6.6	7.1	4.2	1.4	0.9	0.0	0.0
Glucose	4.3	6.3	5.7	4.7	4.3	4.1	3.6	3.1	3.0	2.6
Xylose	2.9	4.5	3.9	3.6	4.0	4.0	4.4	4.8	4.8	3.8
Arabinose	5.5	6.3	6.7	5.9	6.1	7.6	9.3	9.1	6.9	5.7
Acetic acid	0.6	1.0	0.7	1.5	1.3	1.9	3.3	3.5	5.3	6.6
Formic acid	0.0	0.7	0.8	1.4	1.2	1.2	1.7	1.8	2.9	3.5
HMF	0.0	0.0	0.1	0.1	0.1	0.2	0.4	0.4	0.8	1.4
Furfural	0.0	0.0	0.0	0.0	0.1	0.2	0.5	0.7	1.9	2.5
Phenolics	7.6	12.5	9.0	8.0	8.4	6.1	6.3	7.1	7.9	7.7

XOS – xylo-oligosaccharides; GlcOS – gluco-oligosaccharides; AOS – arabino-oligosaccharides; HMF-5-hydroxymethyl furfural; values in parentheses indicate the reaction temperature

The content of oligomers increased with severity until log R_0 of 3.12 but dropped at higher severities. The highest production of total oligosaccharides (24.7 g/L) was achieved at the 190 °C final reaction temperature (log R_0 of 3.12). For the milder conditions, the oligomeric sugars were mainly glucose oligomers (gluco-oligosaccharides, GlcOS), whereas the concentration increased with temperature to reach a maximum (11 g/L) at log R_0 3.57 and then, decreased for the severest conditions. Under this condition, xylan solubilization reached about 80%. These results are in agreement with reported data for corncob Vásquez et al., 2006) and corn straw (Moniz et al., 2013).

A decline of XOS, GlcOS and monosaccharides concentrations and an increase of degradation products can be observed for severities higher than log R_0 3.70. The highest GlcOS concentration of 9.3 g/L was obtained at a final temperature of 190 °C (log R_0 3.12). Hemicellulosic oligosaccharides, such as XOS, also contain arabinose and acetyl groups linked to the xylose backbone, which have been quantified as arabinose oligomers (AOS) and acetyl groups linked to oligosaccharides (AcO). The maximum concentration of AOS, 8.7 g/L, was obtained at log R_0 2.57. AcO reached a maximum of 1.7 g/L at log R_0 3.57. Regarding monosaccharides, arabinose was the most abundant, reaching a concentration of 9.3 g/L (at log R_0 3.57). This arabinose concentration is quite high when compared to the results previously obtained for the autohydrolysis of straws or wood (Garrote et al., 2002; Feria et al., 2011) although arabinose has also been reported as the most important monomeric sugar in the liquors obtained by the autohydrolysis of brewery's spent grains (Carvalheiro et al., 2004). In contrast, the highest xylose concentration was obtained for high severity conditions (log R_0 of 3.7 and 4.0) but decreased thereafter. The glucose concentration was the highest at mild temperatures decreasing for the severest conditions. This significant concentration of glucose and part of arabinose in the hydrolysates might indicate that non-structural carbohydrates are present, namely as extractives. Further characterization of CLR water and ethanol extractives demonstrated the presence of these sugars (data not shown).

Hydroxymethylfurfural (HMF) and furfural that are produced from the degradation of hexoses and pentoses, respectively, increased with increasing temperature. It is possible to observe a higher production of furfural (2.5 g/L) than HMF (1.4 g/L) mainly due to the higher concentration of pentoses. In fact, pentoses are more susceptible to degradation than hexoses (Silva-Fernandes et al., 2015). A similar trend was also found for acetic acid, which arises from the hydrolysis of hemicellulosic acetyl groups (Garrote and Parajó, 2002). The maximum concentration of acetic acid (6.6 g/L) was found at log R_0 4.29. Formic acid is another degradation compound that can appear due to furfural and HMF degradation. Formic acid was detected from a severity condition of log R_0 1.97, reaching 3.5 g/L in the severest condition.

Besides sugars and their degradation products, phenolic compounds were also significant non-sugar compounds in the hydrolysates. In general, these compounds appear in autohydrolysis hydrolysates due to extractive removal from the solid phase and/or solubilization of lignin (Garrote et al., 2003). The concentration of total phenolics obtained from autohydrolysis of CLR was quite high even for moderate temperature conditions. For example, the highest concentration (12.5 g/L) was obtained for log R_0 of 1.97. These values are considerably higher than those reported for corn straw (Moniz et

al., 2013) or feedstock mixtures of eucalyptus residues, wheat straw and olive tree prunings (Silva-Fernandes et al., 2015).

Some studies demonstrated the antioxidant activity of autohydrolysis liquors extracts containing phenolics (Rostro et al., 2014; Akpinar et al., 2010; Conde et al., 2008). For spent coffee grounds, considerable amounts of phenolic compounds in the freeze-dried material recovered after autohydrolysis were reported (82.33–254.00 mg GAE/g LM) and several methods (FRAP, DPPH, ABTS, and TAA) demonstrated the correlation of phenolic compounds to antioxidant activity (Ballesteros et al., 2017). In fact, the antioxidant activity of natural sources offers the possibility of commercial use in the food and cosmetic industries, as an alternative to synthetic antioxidants (Garrote et al., 2003).

The autohydrolysis process was efficient in obtaining of oligosaccharides and soluble sugars, in general at mild temperature, and also interesting for the recovery of the hydrophilic compounds in both mild and severe temperatures, since these fractions can also be valued.

3.3. Composition of the solid phase

Hemicelluloses, cellulose and lignin have different susceptibility to hydrothermal treatments. For the temperature range usually applied (150–230 °C) the most common situation corresponds to a major solubilization of hemicelluloses and a small portion of lignin, together with structural changes of cellulose and minor hydrolysis. The solubilization of lignin is generally higher than cellulose although above 220 °C the cellulose hydrolysis tends to be significant. The solids were chemically characterized after the removal of extractives (Fig. 1).

A progressive solubilization of the hemicellulosic fraction with the temperature increase was observed due to a decrease of xylan, arabinan and acetyl groups. Arabinan was the first component to be completely removed from the solid fraction (at log R_0 of 3.57).

Acetyl groups also decreased with the increase of temperature. The amount of solubilized xylan increased with severity to reach complete solubilization under the most severe condition, showing the efficiency of this treatment to hemicelluloses hydrolysis. Similar results were also obtained for autohydrolysis of other materials, such as olive pruning, corn stover and rye straw (Gullón et al., 2010; Martin et al., 2010; Buruiana et al., 2014). Under the conditions leading to the highest production of XOS (the main oligosaccharides produced) the residual content of hemicellulose was 7.1 (4.1% xylan and

3.0% acetyl groups). Glucans mainly remained in the pretreated solids, ranging between 26.9 and 35.6% and the highest glucan content was obtained for log R_0 4.0. The solubilization of glucan did not increase with the increase of severity and the maximum solubilization occurred at log R_0 of 3.42 (data not shown). The solubilization of glucan is not desired since it should remain in the solid fraction in order to be further hydrolysed with enzymes. This solubilization generally occurs at high operation temperatures. Romaní et al. (2010) reported a decrease of 93% and 79% in the cellulose recovery, respectively, for *Eucalyptus globulus* wood at 240 and 250 °C.

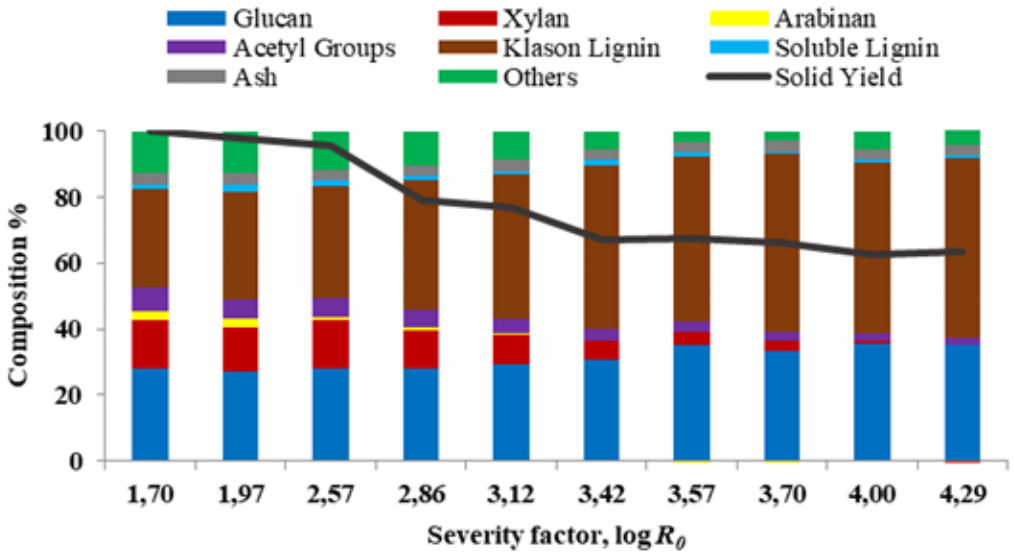

Fig. 1 Composition of *Cistus ladanifer* distillery residues after autohydrolysis under different severity conditions

Klason lignin seems to be not degraded by the hydrolytic treatment and an increase of this component in the solid residue was observed at the most severe conditions, reaching contents up to 54 g/100 g. In all conditions, the lignin yield was always near to 100% of the feedstock lignin. Lignin removal of about 20% or higher have been reported for the autohydrolysis of corncobs and *E. globulus* (Garrote et al., 2002; Roamní et al., 2010). Glucan and lignin together represented about 58 to 90% of the pretreated solids, with the highest value obtained for the most severe conditions. Similar results were reported for rye straw and mixtures of eucalyptus residues, wheat straw and olive tree pruning (Silva-Fernandes et al., 2015).

Pretreatment processes that fractionate biomass to separate hemicelluloses and lignin allow enhanced the hydrolysis of cellulose to produce fermentable sugars, while lignin is also a potential source of diverse products in the framework of biorefineries (Holladay et al., 2007).

The solid residues obtained after the hydrothermal treatment were submitted to successive extractions using solvents with different degrees of polarity and the results of extractives removal are shown in Fig. 2. In fact, these solid residues still contain an important amount of extractives (ranging from 22 to 38%). Water extractives tend to decrease with the increase of severity of the hydrothermal treatments indicating their increasing removal.

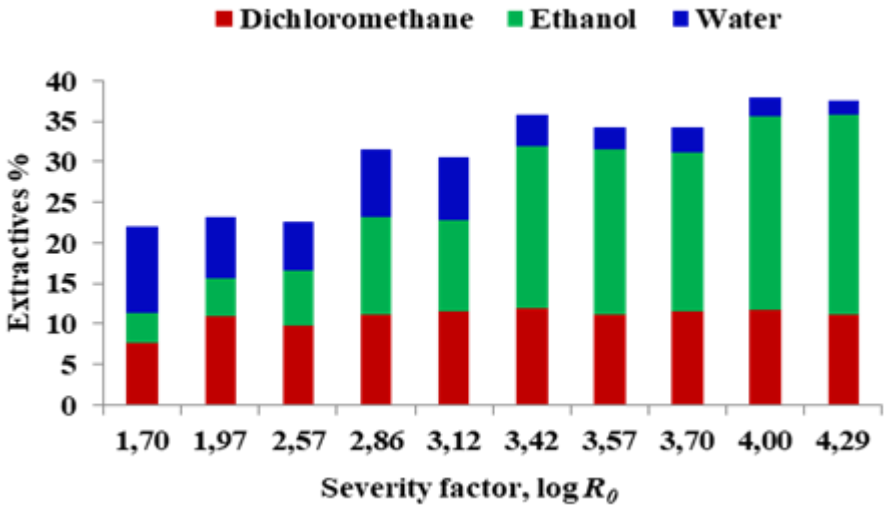

Fig. 2 Effect of autohydrolysis severity conditions on the extractives removal from *Cistus ladanifer* distillery residues

Considering that CLR contains a significant amount of extractives, in this case 8.5% in dichloromethane, 17.2% in ethanol and 16.5% in water, water extractives removal reached 93% for the most severe condition. In contrast, lower amounts of extractives solubilized in ethanol were found for solids obtained at milder conditions but for log R_0 of 3.42 or higher, they increased, representing 57 to 65% of the total extractives remaining in the solid after autohydrolysis. A similar trend occurred with nonpolar extractives, where almost all conditions presented around 11% of dichloromethane extractives. Increases in the extractives content with pretreatment severity have been observed by other authors (Martin-Sampedro et al., 2014; Morais et al., 2016). The partial degradation of some macromolecules during the pretreatments that become extractible in a subsequent solvent extraction could explain this fact. For instance, the partial degradation of polysaccharides, mainly of hemicelluloses renders them readily extractible (Martin-Sampedro et al., 2014).

The aqueous extracts of *C. ladanifer* have been described as rich in phenolic compounds and their antimicrobial, cytotoxic and antioxidant capacity have already been evaluated (Barrajón-Catalán et al., 2010; Fernandez-Arroyo et al., 2010; Barros et al., 2013). *C. ladanifer* fresh leaves had a total amount of phenolic compounds of 38.44 mg/g

extract, including phenolic acids and its derivatives, ellagic acid derivatives and flavonoids (Barros et al., 2013).

Also the determination of extractives in hydrothermal processed solids showed that this is an efficient treatment to remove polar extractives (solubilized in water) at high temperature but not for nonpolar compounds. Nevertheless, milder temperatures were more efficient in the removal of total extractives. This important amount of extractives suggests an additional potential for valorization these residues.

4. Conclusions

Non-isothermal autohydrolysis of CLR demonstrated to be efficient for the hydrolysis of hemicelluloses and for the production of oligosaccharides (maximum of 24.7 g/L). Besides these, an important production of phenolic compounds (6.1–12.5 g/L), associated to the extractive fraction, was also obtained, suggesting the potential of these liquors for functional applications, i.e. bioactive. This, together with the consequent enrichment of the solid phase in glucan and lignin, highlights the relevance of CLR as an interesting feedstock option for valuable co-production of biofuels and bioproducts solutions for biorefineries.

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CAPÍTULO V

Distillery residues from *Cistus ladanifer* (rockrose) as feedstock for the production of added-value phenolic compounds and hemicellulosic oligosaccharides

Este capítulo tem como base a seguinte publicação:

Júnia Alves-Ferreira, Luís C. Duarte, Ana Lourenço, Luísa B. Roseiro, Maria C. Fernandes, Helena Pereira, Florbela Carvalheiro (2019). Distillery residues from *Cistus ladanifer* (rockrose) as feedstock for the production of added-value phenolic compounds and hemicellulosic oligosaccharides BioEnergy Research, 12, 347–358.

Abstract

Cistus ladanifer residues obtained after essential oil distillation were extracted with ethanol and water (CLRext) and subsequently hydrothermally treated (autohydrolysis) in order to selectively hydrolyze hemicelluloses. The extraction removed a significant amount of potentially valuable compounds (40% w/w, dry basis), foremost, phenolic compounds (0.250 and 0.363 g). gallic acid equivalent/g extract, respectively, for water and ethanol). Autohydrolysis was studied under diverse severity factors (log R_0), in the temperature range of 150 to 230 °C. The hydrolysates mainly contain oligosaccharides, reaching the highest concentration (23.5 g/L) for log R_0 of 3.07 (190 °C), corresponding to a yield of 15 g oligosaccharides/100 g dry feedstock. The processed solids are enriched in glucan and lignin. The maximum glucan content (35%) was attained at log R_0 of 3.51 (205 °C). Py-GC/MS confirmed the reduction of pentose-derived carbohydrates in the solid after hydrothermal treatment and an increase of syringil units in the lignin compared to the untreated biomass. These results show the potential use of this *C. ladanifer* residue for the production of phenolic extracts, and hemicellulosic oligosaccharides, together with the production of a cellulose- and lignin-rich solid stream.

Keywords: Extraction, Fractionation, Hemicelluloses, Hydrothermal treatment, Phenolic compounds, Pretreatment

1. Introduction

Rockrose (*Cistus ladanifer*) is one of the most significant natural shrubs in the Mediterranean basin, mainly distributed in countries such as Portugal, Spain, Greece, Italy, Algeria, and Morocco (Mariotti et al., 1997; Weyerstahl et al., 1998). It is estimated that it extends by an area of ca. 2 million hectares in the south/southwest of the Iberian Peninsula. Specifically, in Portugal, it extends throughout the country, especially in the center and south regions (Pérez et al., 2011; Clamote et al., 2019). This plant shows high proliferation, occupying abandoned or unmanaged agricultural areas and may represent a fire hazard in some regions. It is generally recognized that, if not properly controlled, this species is one of the major responsible for fire spreading in the Mediterranean forest ("Montado"), as allegedly, it has some evolutive traits that enable it to survive and rapidly colonize burnt areas (Tárrega et al., 2001; Calvo et al., 2005; Delgado et al., 2008). Although *C. ladanifer* is being explored industrially (Andrade et al., 2009) as a source of resin (labdanum gum) and essential oils for cosmetic and perfume industries, it is estimated that the global use of *C. ladanifer* is only about 10,000 tons per year, with 80% of the products obtained coming from Spain, mainly from Andalusia (Biolandes, 2019). As such, this feedstock can be considered still to be underexplored, especially the biomass remaining after the steam distillation used for essential oil production, which is used only for energy production by combustion, a very low-value application. As the processes used for the essential oil extraction are relatively mild, i.e., aqueous processes under moderate temperature conditions, the residual lignocellulosic materials retain much of their original, structural and non-structural, chemical components, and maintain their potential for the production of a wide range of co-products. Among the structural component–derived products, xylan-derivatives, e.g., xylo-oligosaccharides (XOS), are the ones with higher market value due to their bioactive properties that make them interesting as food and pharmaceutical ingredients (www.transparencymarketresearch.com). Phenolic compounds, either derived from non-structural components and lignin, can also find applications in pharmaceutical and food industries that are expected to have very significant growth rate until 2025 (Ahuja & Deb, 2008; www.grandviewresearch.com).

In fact, the development of effective valorization routes for all chemical fractions from forest understory biomass can contribute to its sustainable management, and strength both the environmental security and local economy. The use of the rockrose, for example, has a great socioeconomic importance for some Iberian communities since it is the base of several small-scale extraction industries with a great impact on the local economies (Morgado et al., 2005). In this perspective, the use of rockrose not only as a source to obtain essential oils or labdanum gum but also as a source of other products,

namely from its industrial residues, may be strategic in the expansion of distilleries and consequently in the development of rural communities. Thus, the optimization of integrated processes for biomass utilization in a biorefinery framework with lower capital expenditures is important for these rural areas where endogenous biomass potential is still poorly explored.

Nevertheless, most studies on this species have been only related to ecological and environmental issues or to the composition and bioactivity of its essential oils and extracts (Chaves et al, 1997; Sosa et al., 2005; Bouamama et al., 2006; Delgado et al., 2008; Narbona et al., 2010; Verdeguer et al., 2012). Only few studies suggest the use of *C. ladanifer* as a raw material for the production of biofuels (e.g., ethanol) or bioproducts (Gil et al., 2012; Ferro et al., 2015; Fernandes et al., 2018). In our previous studies, *C. ladanifer* distillery residues (CLRs) were chemically characterized and pointed as an interesting residual biomass to be upgraded in a biorefinery framework (Alves-Ferreira et al., 2017). However, application of *C. ladanifer* at field scale requires further investigation to lead to an integrative profit of native biodiversity and to the economic development in rural and urban unusable areas (Raimundo et al., 2018).

Biomass fractionation in a biorefinery may include different processes. The removal of extracts may be attained using water and solvents such as ethanol, acetone, and ethyl ether. In the pretreatment for hemicellulose hydrolysis, hydrothermal processes such as liquid hot water (LHW) are relevant sustainable options that use compressed liquid water and heat and do not require the addition of chemicals. Typically, under these conditions, there is a low formation of degradation compounds, while cellulose and lignin are almost not degraded (Carvalheiro et al., 2009) and can be used in further applications. The use of water as the only added chemical decreases reactor construction costs due to low-corrosion potential. Furthermore, as the steps of neutralization, catalysts recycling and precipitates removal are not necessary, this evidences the advantages of LHW and its cost-saving potential compared to other hydrolytic technologies. On the other hand, the use of large volumes of water and energetic requirements is, unfortunately, some of the disadvantages of the process (Alvira et al., 2010). LHW processes have been applied to different types of biomass, such as *Pinus radiata* wood (Santos et al., 2018), corncobs (Moura et al., 2007), cereal straws (Carvalheiro et al., 2009; Moniz et al., 2013; 2014), and olive tree prunings (Cara et al., 2012). The results obtained with the hydrothermal treatments depend on the biomass type and composition used as feedstock.

In a previous work (Alves-Ferreira et al., 2017), it was shown that direct hydrothermal processing of *C. ladanifer* enables a high production of oligosaccharides, although an important production of other compounds, e.g., monosaccharides, was also obtained. Furthermore, *C. ladanifer* biomass residues also contain a relevant content of

extractives with potential functional applications. Although these compounds can be partially extracted during the hydrothermal process, they will decrease both the purity of the oligosaccharide-rich stream and, due to potential reactions during autohydrolysis, that decrease their value. Hence, the dedicated extraction and the subsequent hydrothermal fractionation of extracted biomass may increase the number of output products and their purity.

This work aims to develop an integrated fractionation process, to selectively separate the extractives and other potential value-added fractions, including the hemicellulosic sugars, and obtain a solid fraction containing cellulose and lignin. It is also the goal of this work to contribute to the development of small-scale and locally adapted biorefineries that will strengthen local economies.

2. Material and methods

2.1. Preparation and physical characterization of raw material

The *Cistus ladanifer* residues (CLRs) were obtained from a commercial essential oil distillery located in the Alentejo region (Portel, Portugal). These residues include stems, bark, leaves, and cysts that were previously extracted by steam distillation to produce essential oils. The CLR material was then dried and milled as described before (Alves-Ferreira et al., 2017). A granulometric characterization of the milled CLR was performed using a sieve shaker (Endecotts, England) with sieves of different size pores (0.25, 0.5, 0.71, 1, 1.6, and 2.36 mm). The milled raw material (ca. 100 g) was screened, in triplicate, for at least 20 min, and the material retained on each sieve was weighed for the determination of the respective mass fraction.

2.2. Extraction of raw material

The extraction of CLR was performed in a semi-pilot Soxhlet system (2 L) using 0.4 kg of biomass that was consecutively extracted with ethanol (60 h, 1 cycle/h) and water (96 h, 0.35 cycle/h) based on NREL protocol (Sluiter et al., 2008). Extractive quantification varied less than 5% among the runs. After extraction and drying (40 °C), the material was carefully homogenized in order to obtain a uniform lot. The extracted CLR used in the remaining of this work as feedstock was coded as CLRext.

2.3. Hydrothermal processing

The hydrothermal treatment (autohydrolysis) of CLRext was carried out using a 600-mL stainless steel reactor (Parr Instruments Co). The raw material was mixed with water with a 6:1 liquid-to-solid ratio (w/w), and the agitation speed was set at 150 rpm, as previously described by Alves-Ferreira et al. (2017). A defined heating rate from 100 °C was imposed to the system (5.0 ± 0.1 °C/min), which was rigorously maintained among runs. Once the desired final temperature (ranging from 150 to 230 °C) was attained, the reactor is rapidly cooled down to room temperature, reaching 100 °C in less than 3 min. The reactor is open and the solid and liquid phases were separated using a hydraulic press. The recovered solids were washed with the double of distilled water used in the hydrothermal pretreatment and dried at 50 °C by approximately 48 h and dried for another 48 h at room temperature. The recovered liquid phase was filtered (quantitative filter paper, Filter-Lab no. 1235) and stored at 4 °C until further analysis. Both the liquid and solid phases were further characterized as described below.

The effects of the time and temperature profile of the non-isothermal process were computed using the following equation for the severity factor (log R_0) (Overend & Chornet, 1987):

$$R_0 = \int_0^t exp(\frac{T\,(t) - T_{ref}}{14.75})dt$$

where T (°C) is the temperature as a function of time t (min), T ref is the reference temperature (100 °C), and the value 14.75 is an empirical parameter related to the activation energy. The contribution of the cooling period was negligible for all conditions.

2.4. *Analytical methods*

2.4.1. Chemical characterization of the solids

The feedstock used (CLRext) and the solid residues obtained after the hydrothermal treatments were chemically characterized for carbohydrate content, Klason lignin, protein, and ash. After milling to a particle size smaller than 0.5 mm, the moisture content was calculated by oven-drying at 105 °C to constant weight. Ash content was determined using NREL/TP-510-42622 protocol (Sluiter et al., 2005).

For the quantification of macromolecular compounds, the samples were sequentially hydrolyzed with 72% (w/w) H_2SO_4 and 4% (w/w) H_2SO_4. The amounts of glucan, xylan, arabinan, and acetyl groups were determined from the corresponding monomers (see below). The acid-insoluble residue was considered as Klason lignin, after correction for ash. The acid-soluble lignin was determined in the filtrate by UV spectroscopy at 206 nm using 110 L/(g cm) as absorptivity (extinction coefficient), as suggested (TAPPI UM-250). The determination of protein was carried out according to the Kjeldahl method (AOAC, 1975) using the N × 6.25 conversion factor.

The calculations used for the yields of the structural component remaining in the solid phase after treatments, conversions into oligosaccharides, monosaccharides, and degradation compounds in g/100 g of feedstock, and the percentage of solubilization were calculated as reported by Carvalheiro et al. (2004).

All analyzes were carried out, at least, in duplicate. Variation among replicates was always below 5%, and hence, the results are mainly presented without standard errors in order to increase tables readability.

2.4.2. Chemical characterization of extractives and hydrolysates

The hydrolysates that resulted from autohydrolysis were analyzed by HPLC for sugars, aliphatic acids, and furan derivatives. The quantification and characterization of oligosaccharides were carried out, at least in duplicate, by post-hydrolysis with 4% (w/w) H_2 SO_4 (Moniz et al., 2013), and the content of the oligosaccharides was calculated by the difference between the monosaccharides present in the post-hydrolysates and in the hydrolysates, taking into account the corresponding dilution and sugar degradation.

The quantification of sugar monomers (xylose, glucose, and arabinose), acetic acid, and other sugar-derived compounds (levulinic acid, formic acid, furfural, and HMF) was carried out in a HPLC equipped with both an RI and diode-array detector (DAD), using an Aminex HPX-87H column (Bio-Rad, USA). The chromatographic analysis was carried out at 50 °C using sulfuric acid (5 mM) as eluent, at 0.6 mL/min flow rate.

The total phenolic content in the extractives or in hydrolysates was quantified by the Folin–Ciocalteu colorimetric method (adapted from Roseiro et al. 2013). Briefly, 100 µL of the extract (or water for blank) was added to 250 µL of the 1:1 (v/v) diluted Folin–Ciocalteu reagent, 1250 µL of 20% (m/v) Na_2CO_3 and mixed with 400 µL of Milli Q water. Absorbance was measured at 750 nm using 250 µL of the mixture in a microplate spectrophotometer (MultiskanTM GO, Thermo Scientific, USA) after 40 min incubation at

room temperature. Gallic acid was used as standard and total phenolics concentration was expressed as gallic acid equivalents (GAEs).

The phenolic profiles of the 205 °C and 230 °C treatment hydrolysates were obtained by capillary zone electrophoresis (CZE), using an Agilent system, equipped with a DAD. CZE separation was performed using a fused-silica uncoated capillary with extended light path (i.d. 50 µm, 62/56-cm length). The samples were injected at 50 mbar for 6 s and under a voltage of 30 kV. The temperature was maintained at 25 °C. The electrolyte (15 mM borate in 10% MeOH) was adjusted to pH 9.1. The capillary was pre-conditioned by flushing with 0.1 M NaOH for 3 min and subsequently running buffer for 3 min. Compounds were detected at 200 nm and identified by the comparison of their UV spectra and migration times to authentic standards.

All analytical determinations using HPLC and CZE were carried out, at least, in duplicate.

2.4.3. Analytical pyrolysis

Pyrolysis-gas chromatography-mass spectrometry (Py-GC/MS) analysis was performed in a 5150 CDS apparatus linked to an Agilent GC 7890B coupled to a mass detector system 5977B using electron impact mode (EI at 70 eV). The material (approximately 100 µg) was pyrolyzed at 550 °C for 10 s. The injector temperature was kept at 270 °C and the GC/MS interface was set at 280 °C.

The carrier gas, helium, flowed at a rate of 1 mL/min to elute the sample into a fused capillary column ZB-1701 (60 m × 0.25 mm i.d. × 0.25 µm film thickness). The column temperature was kept at 40 °C for 4 min., raised to 70 °C at a rate of 10°C/min, raised to 100 °C at 5 °C/min, then to 265 °C at 3 °C/min (held for 3 min), and to 270 °C at 5 ° C/min (held for 9 min). The identification of the compounds was performed by the comparison with Wiley, NIST2014 computer libraries, and literature (Faix et al., 1991; Ralph and Hatfield, 1991), being the percentage of each compound calculated according to the total area of the chromatogram.

3. Results and discussion

3.1. Particle size distribution

The granulometric separation after size reduction to particles smaller than 6 mm of the *C. ladanifer* distillery residues (CLRs) was carried out. The CLR biomass was fractionated with little formation of both fines (the < 0.25 mm fraction corresponded to 7.3% of the total) and of larger fractions (the ≥ 1.6 mm fractions represented 14.4% of the total). The major fractions corresponding to 67% of the total mass had particle sizes ranging from 0.5 to 1.6 mm.

The mechanical fractionation, i.e., the granulometric distribution of the granulates, depends on the biomass type, namely of its structural and physical characteristics. Similar granulometric distribution was reported by Moniz et al. (2013) for the fines particles (< 0.25 mm) of corn straw, while the granulation of tree barks varies with the species (Miranda et al., 2012; 2013; Baptista et al., 2013).

Taking into account the fractionation pattern of the CLR biomass it was decided that no separation was needed before the hydrothermal processing (e.g. separation of fines) and all the fractions were used.

3.2. Chemical composition of Cistus ladanifer residues

The *C. ladanifer* residue originating from essential oil distillation was previously chemically characterized (Alves-Ferreira et al., 2019) and shown to have a significant amount of extractives (ca. 42% of total extracts using dichloromethane, ethanol, and water), indicating an important potential for their valorization (Alves-Ferreira et al., 2017). Therefore, an extraction using successively ethanol and water was carried out before the hydrothermal treatment to remove the major part of soluble compounds, namely the polar compounds. The extraction with dichloromethane was not carried out due to the low content of non-polar compounds as well as a way to reduce the extraction costs and increase industrial security. The extraction with ethanol and water removed about 37% of extractives (13.6% in water and 23.3% in ethanol) (Table 1). Ethanol extracts are higher than the values usually reported for these materials. Sánchez-Vioque et al. (2013) reported a content of 10.6 g dry extract/100 g dry material for the residues of *C. ladanifer* from the essential oil industry extracted with ethanol in a Soxhlet apparatus. A content of ethanol and acetone extractives around 8.5% and 14.2%, respectively, in non-distilled plants, was previously reported (Andrade et al., 2019).

Table 1. Extractives content of *C. ladanifer* residues (CLR) and chemical composition of the extracted *C. ladanifer* residues (CLRext)

	% od mass
Total extractives	36.9±0.4
Ethanol	23.3±0.8
Water	13.6±1.2
	CLRext[a] % od mass
Cellulose (as glucan)	27.8±0.5
Hemicelluloses	22.0±0.3
Xylan	15.7±0.1
Arabinan	3.6±0.2
Acetyl groups	2.8±0.1
Klason lignin	29.4±0.3
Soluble lignin	2.9±0.1
Protein	7.3±0.4
Ash	4.2±0.2
Others (by difference)	7.4±0.5

[a] Extractive free

The ethanolic extracts showed higher total phenolic content compared with the water extracts (respectively, 0.363 *vs*. 0.250 g GAE/g extract, corresponding to 6.2 *vs*. 4.4 g GAE/100 g dry weight of the original biomass). These results are above those detected by Andrade et al. (2009) for ethanolic extracts (0.255 g GAE/g extract). Tomás-Menor et al. (2013) found a range of 9.9 to 13.3 g GAE/100 g dry weight of *C. ladanifer* using various extraction and drying techniques. Barrajón-Catalán et al. (2010) also determined high values of phenolics, but for aqueous extracts of the leaves (about 23 g GAE/100 g dry weight).

The phenolic profile of water and ethanol extractives shows the presence of the flavonoids (apigenin, rutin), other non-identified phenolics with characteristic flavonoid spectra, and gallic acid (Fig. 1). Besides other gallic acid and ellagic acid, flavonoids (kaempferol methyl ether, kaempferol dimethyl ether, kaempferol diglucoside, apigenin) were the predominant phenolic compounds found by Sánchez-Vioque et al. (2013) in ethanolic extracts of CLR. The bioactivity of *C. ladanifer* extracts has been described in the literature, and significant antioxidant and antimicrobial capacities, as well as cytotoxic activity against cancer cells associated to the presence of polyphenols, tannins, and flavonoids (Andrade et al., 2009; Barrajón-Catalán et al., 2010; Tomás-Menor et al., 2013), were the main activities reported.

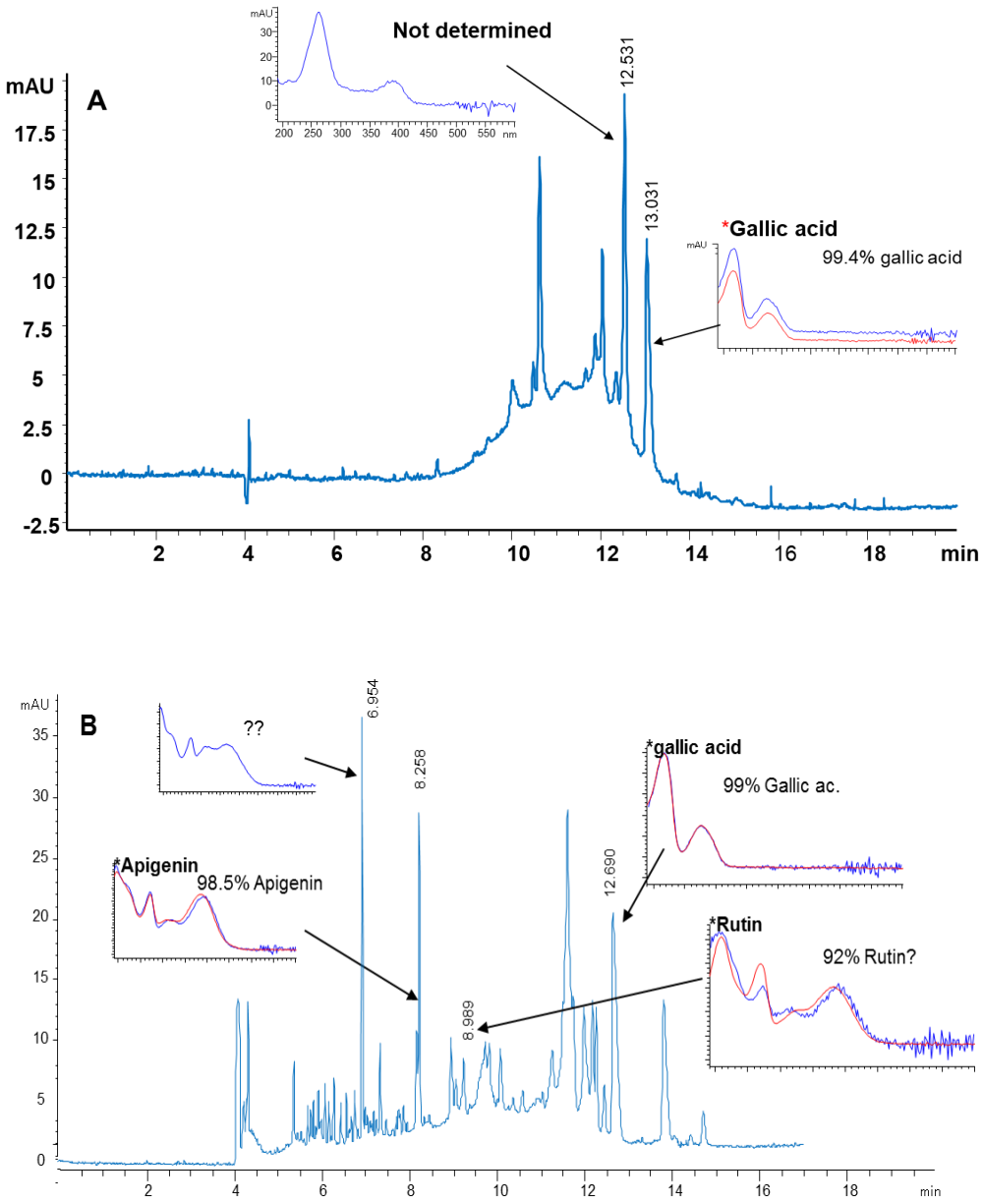

Fig. 1 Electropherogram for the phenolic profile of water- (A) and ethanol (B) extractives obtained from *C. ladanifer* residues. Matching percentage was obtained by the comparison of the spectra with the spectra of authentic standards run at the same analytical conditions

The composition of the extracted CLR used in the hydrothermal treatment (CLRext) is shown in Table 1. The extracted material contained 29.4% lignin, 27.8% glucan, 15.7% xylan, 3.6% arabinan, and 2.8% acetyl groups. The available data from other works show that the chemical composition of CLR, in particular the content of extractives and Klason lignin, strongly depends on the plant age and varies between organs and their proportion in the sampled biomass (Andrade et al., 2009; Gil et al., 2012; Ferro et al., 2015; Alves-Ferreira et al., 2017; Fernandes et al., 2018). Gil et al. (2012) found similar results to this work for Klason lignin (ca. 30%). Fernandes et al. (2018) and

Ferro et al. (2015) reported values of 41.5–42.2%, 15.6–32%, and 6.2–16.7%, respectively, for polysaccharides, Klason lignin, and extractives of 10-year-old plants. Comparing to other lignocellulosic residues, the polysaccharides content of CLRext was similar to the values reported for vine pruning and olive tree pruning residues (Romero et al., 2017; Surek & Buyukkileci, 2017).

Before extraction, CLR present ca. 30% of total carbohydrate, being that after extraction, this content is ca. of 50%. Thus, the extraction with ethanol and water, besides allowing the separation of the extractives fractions, enables the increase of polysaccharide content of the samples to be used in fractionation processes on a feedstock mass unit basis, which is an advantageous trait.

3.3. Autohydrolysis profile

The impact of autohydrolysis as a function of the severity factor (log R_0, 2.01–4.25) on the chemical components of the feedstock is shown in Fig. 2a-c by plotting the xylan, glucan, and Klason lignin in the solid and the oligosaccharides, monosaccharides, and by-products, showing the recovery of these components in the solid phase and in the liquid phase as mass yield in relation to the feedstock (CLRext).

Xylan hydrolysis was significant starting at a severity factor of 2.89, reaching 91.6% of solubilization at the severest condition assayed. The maximum conversion of xylan into xylo-oligosaccharides (XOS) was found for a log R*o* of 3.51, corresponding to 64.8% of xylan (10.2 g/100 g feedstock, Fig.2a) and when 61.8% of the original xylan was solubilized. In these conditions, 72.6% of xylan was recovered as soluble saccharides (XOS and xylose) in the hydrolysate. Other works have presented comparable XOS yields from several species, i.e., Hazelnut shell (62%, (Jesus et al., 2017) and vine pruning residue (63.7%, (Surek & Buyukkileci, 2017)) but higher than the reported for rice husks (30%, (Nabarlatz et al., 2007)).

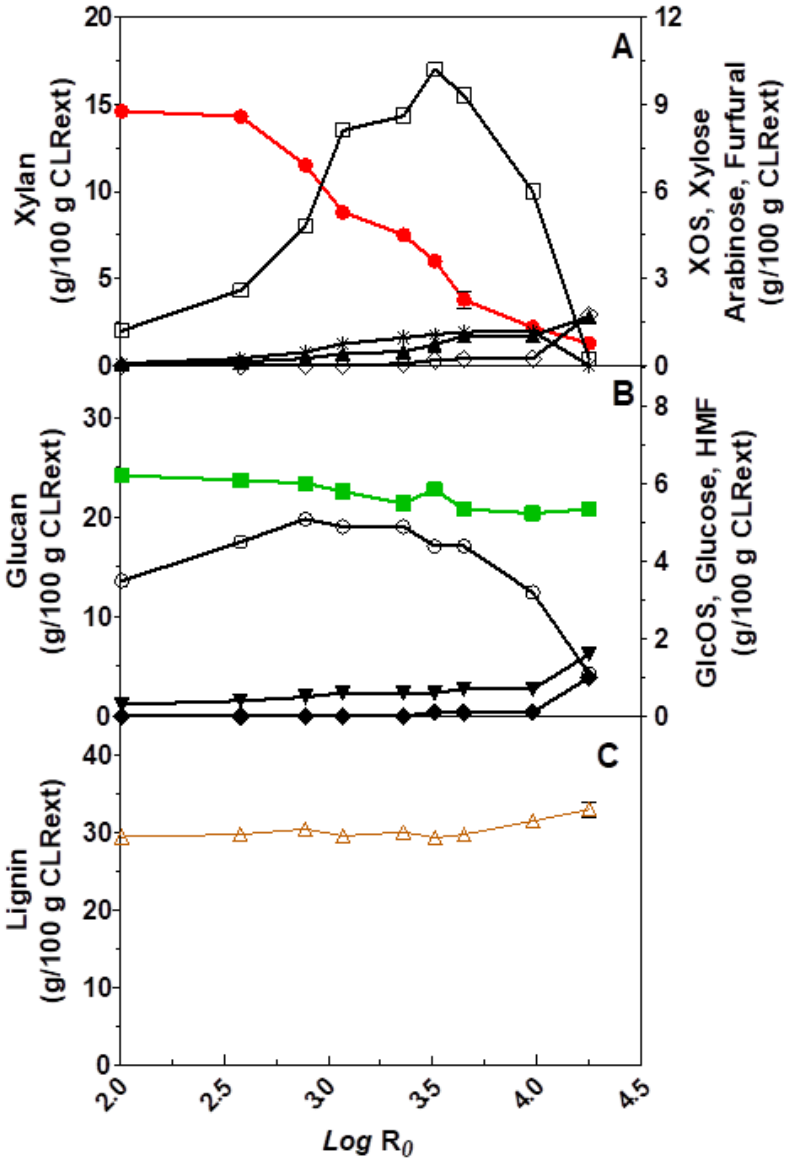

Fig. 2 (A-C) Recovery of components (g/100 g feedstock) after autohydrolysis of *C. ladanifer* residues as function of severity. A: Xylan (●) in the solid and xylo-oligosaccharides (□), xylose (▲), arabinose (*), and furfural (◊) in the liquid; B: Glucan (■) in the solid and gluco-oligosaccharides (○), glucose (▼), and hydroxymethylfurfural (♦) in the liquid; C: Klason lignin in the solid (Δ).

Xylose and arabinose were obtained with maximum yield in the hydrolysate for severity factors of 4.25 and 3.98, respectively, reaching a sum of 4.7 g/100 g feedstock. Arabinose concentration increased with severity, but it was not detected at the most severe condition, most probably due to degradation reactions. In contrast, xylose steadily increased with severity. Other authors have observed a sharp decrease in xylose content in the hydrolysate at severe conditions (240 °C) (Carvalheiro et al., 2004; Moniz et al., 2013), together with an increase of furfural. Furfural was found here in the hydrolysates

only starting from log R_0 = 3.65 reaching only 18.2% of the initial xylan for the severest condition.

In contrast to xylan, glucan is mainly retained in the solid phase, as it was only partially depolymerized (Fig. 2b). The maximum removal of glucan occurred at log R_0 = 3.98 with 26.6% solubilization of the original glucan. This value is slightly higher than the usually reported for other materials. Furthermore, the glucan profile obtained in this work differs from the most common obtained for the autohydrolysis of lignocellulosic biomass, as typically a relevant increase of glucan content is usually observed. This can be associated to the glucan composition of the feedstocks as a similar profile was obtained for the autohydrolysis of brewery's spent grain (Carvalheiro et al., 2004). The highest yields of gluco-oligosaccharides (GlcOS) was achieved at log R_0 2.89 although almost a plateau can be observed between this severity and log R_0 3.36, obtaining approximately 5 g/100 g feedstock. The maximum yield of glucose, 1.6 g/100 g feedstock, was obtained with the severest condition and corresponded to 5.7% of the initial glucan. In all conditions, the HMF concentrations increased with temperature, reaching at a maximum value of 1.0 g/100 g feedstock.

Figure 2c shows the effect of autohydrolysis on Klason lignin yield. Lignin seems not to be degraded by the hydrolytic treatment and there was even a small increase in the lignin yield obtained at the severest conditions, conversely to the described for other materials, e.g., pine wood (Santos et al., 2018) and wheat straw (Carvalheiro et al., 2009). Nevertheless, the behavior observed here has also been described for other materials and was explained as a consequence of condensation reactions between lignin and other (degradation) products/compounds (e.g., protein) forming an insoluble product usually called pseudo-lignin (Heitz et al., 1991; Carvalheiro et al, 2004; Rasmussen et al., 2014).

3.4. *Chemical characterization of soluble products*

The main non-volatile components present in the liquid stream are sugars, mostly in the oligomeric form. As autohydrolysis mainly affected hemicelluloses, the sugars are mostly (arabino) xylooligomers (Table 2), with glucooligomers appearing in relatively higher concentration from the start of the process, indicating their origin from easily accessible/hydrolysable glucan. Total oligosaccharide concentration reached a maximum of 23.5 g/L (14.8 g/100 g) of CLRext yield at log R_0 of 3.07 (190 °C) but dropped to 2.3 g/L at log R_0 = 4.25 (maximum temperature). In the conditions leading to the highest oligosaccharide production, non-volatile components represent 62% of the total dissolved solids. The concentration of XOS progressively increased up to a maximum of 16.0 g/L (at

log R_0 = 3.51); after which, there was an accentuated decrease until 0.3 g/L at log R_0 = 4.25 (230 °C). The maximum concentration of arabinose containing oligomers (AOS), 3.9 g/L, was found at log R_0 of 2.89 (180 °C), lower than for the XOS, clearly indicating a higher rate of removal of the arabinosyl branches. The reported concentrations are above those obtained for hydrolysates of other lignocellulosic materials such as wheat straw or mixtures of forest and marginal land resources (Carvalheiro et al., 2009; Pontes et al., 2018), which is in agreement with the slightly higher arabinan content of this feedstock but also due to the higher solid loading used in this work. Indeed, hydrolysates of unextracted CLR had still higher values of GlcOS and AOS, and lower values of XOS compared to CLRext hydrolysates (Alves-Ferreira et al., 2017). In the unextracted CLR hydrolysates, those GlcOS and AOS can be partially obtained from the extractives. It is also remarkable that CLRext can produce higher ratio of XOS/total oligosaccharides, as these compounds are potentially the most relevant as bioactive oligosaccharides in hydrolysates (Aachary & Prapulla, 2011).

Table 2. Composition (g/L) of the liquors obtained from the autohydrolysis of the extracted *C. ladanifer* residues for the severity conditions tested

Log R_0	Oligosaccharides					Monosaccharides			Other products					
	XOS	AOS	GlcOS	Total	AcO	Xylose	Arabinose	Glucose	Acetic acid	Formic acid	Levulinic acid	Furfural	HMF	Total phenolics
2.01	2.0	3.50	5.8	11.3	0.5	0.1	0.20	0.6	n.d.	n.d.	n.d.	n.d.	n.d.	2.4
2.58	4.2	3.80	7.3	15.3	1.1	0.4	0.70	0.7	0.1	0.5	n.d.	[a]	[a]	1.7
2.89	7.8	3.90	8.3	20.0	1.7	0.7	1.40	0.9	0.3	0.8	n.d.	[a]	[a]	2.4
3.07	12.9	2.90	7.8	23.5	2.8	1.2	2.30	1.0	0.6	1.2	n.d.	[a]	[a.]	2.4
3.36	13.7	2.00	7.7	23.4	2.9	1.5	2.90	1.1	0.8	1.1	n.d.	[a]	[a]	2.3
3.51	16.0	0.40	7.0	23.4	3.2	2.2	3.40	1.0	1.4	1.3	n.d.	[a]	0.1	2.8
3.65	14.5	n.d.	6.9	20.7	3.0	3.0	3.50	1.2	2.3	1.7	0.4	0.4	0.1	3.5
3.98	9.4	n.d.	4.9	12.2	3.8	3.1	3.50	1.3	2.4	1.8	0.5	0.4	0.2	3.7
4.25	0.3	0.30	1.7	2.3	n.d.	5.0	n.d.	2.8	7.0	4.7	1.3	3.3	1.2	4.4

[a] Below the quantification limit (< 0.04)

n.d., not detected; XOS, xylo-oligosaccharides; AOS, arabino-oligosaccharides, gluco-oligosaccharides; AcO, acetyl groups linked to oligosaccharides; Xyl, xylose; 5-hydroxymethyl furfural

The main monomeric sugar found was arabinose, except for the severest condition, where it was surpassed by xylose due to the degradation of XOS into xylose and arabinose into furfural. Nevertheless, the total monosaccharide content reported here was significantly lower than the data reported in the liquid phase of unextracted CLR (Alves-Ferreira et al., 2017) which is also an advantage when using these hydrolysates as potential bioactive (e.g., prebiotic) products.

The increase of acetic acid concentration is associated to the hydrolysis of hemicelluloses; the maximum concentration (7.0 g/L) occurred at log R_0 = 4.25, corresponding to 4.5 g/100 g raw material. This value, higher than the potential acetic acid present in the feedstock, may suggest other degradation reactions associated to acetic acid formation. The formation of acetic acid and other organic acids due to xylose degradation with temperature increase was previously reported by Oefner et al. (1992). However, as the acetic acid increase found in this work was much higher than that reported by Oefner et al. (1992), it is possible that an overlap of an unknown compound has occurred.

Regarding degradation products, furfural, HMF, formic acid, and levulinic acid, it is important to note that the concentration of furfural that was detected for 210 °C or higher temperatures is almost three times higher than HMF. This is mainly due to the higher amount of solubilized pentoses in relation to hexoses. The maximal HMF concentration was 1.0 g/L and corresponded to 3.4% of the initial glucan. The maximal concentration of formic acid (4.7 g/L) was obtained at log R_0 = 4.25; levulinic acid was not detected up to log R_0 of 3.51 (205 °C) and achieved the highest concentration (1.3 g/L) at the highest temperature assayed.

Furan derivative concentrations obtained from the hydrolysis of unextracted CLR (Alves-Ferreira et al., 2017) were above those presented by CLRext in almost all conditions except in the most severe one. The presence of these compounds is undesired when the aim is to produce oligosaccharide mixtures with potential bioactive properties or hydrolysates for fermentation media.

Regarding phenolic compounds, their concentration was highest at log R_0 of 4.25 (4.4 g/L, corresponding to 2.83 g/100 g CLRext). Although no apparent solubilization of lignin seems to occur (see below), phenolic compounds obtained may be mainly lignin-derived products and/or derived from the solubilization, at higher temperatures, of the remaining non-polar compounds that were still present in the feedstock. Nevertheless, the phenolic compounds present are lower than the obtained from unextracted CLR (Alves-Ferreira et al., 2017).

CZE profiles of the hydrolysates from the severest condition of the hydrothermal treatment of CLRext show furfural as the main component, followed by gallic acid and

catechin hydrate (Fig. 3). This confirms the data obtained for the chemical characterization of the hydrolysates that indicate the higher levels of furfural and phenolic compounds mainly at the maximum temperature. Ellagitannins, in particular punicalagin derivatives, have been reported as the major compounds found in the aqueous extract of the aerial part of *C. ladanifer*, e.g., 0.24% for gallic acid and 3.5% (w/w) for all ellagitannins (Barrajón-Catalán, et al., 2010). Phenolic compounds such as ferulic acid, p-hydroxybenzoic, vanillic, p-coumaric, and caffeic acids and terpenes have also been detected in the *C. ladanifer* aqueous extracts (Herranz et al., 2006).

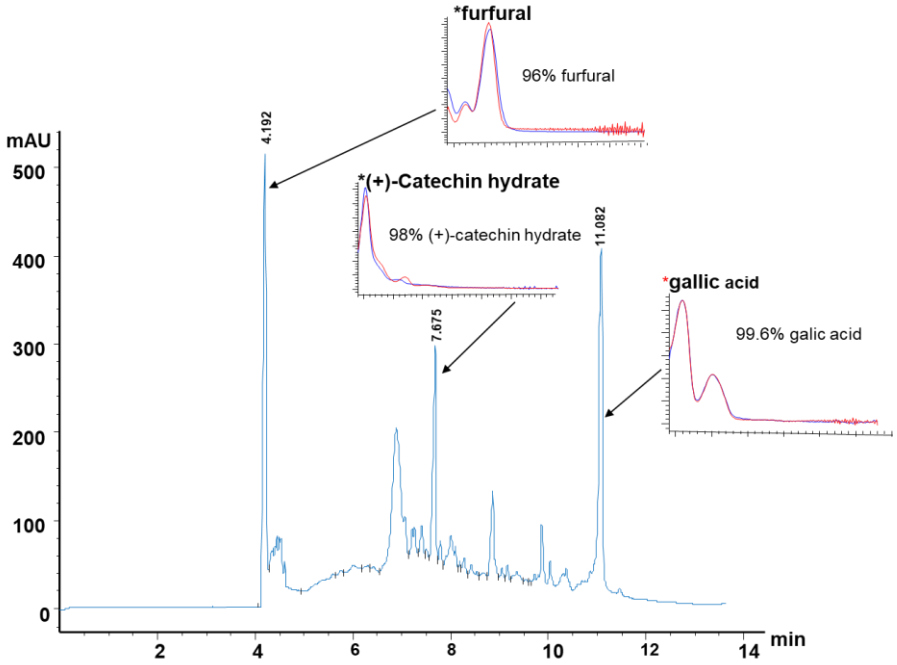

Fig. 3 Electropherogram for the hydrolysate obtained at the severest condition (log R*o* = 4.25, 230°C) of the hydrothermal treatment of *C. ladanifer* residues. Matching percentage was obtained by the comparison of the spectra with the spectra of authentic standards run at the same analytical conditions

3.5. Chemical characterization of processed solids

The chemical composition of the solid fraction obtained after solvent extraction and hydrothermal treatments is shown in Table 3.

The yield in solids decreased sharply with temperature down to 61.3% at log R_0 of 3.65 and stabilized thereafter. Hemicellulose solubilization is the main reason for the solid mass loss. As expected, the contents of arabinan and acetyl groups decreased and no arabinan was found in the solids obtained from 190 °C (log R_0 of 3.07), and the acetyl groups were removed up to log R_0 of 3.98. Xylan was not fully removed by the

hydrothermal treatment and solubilization ranged from 6.9 to 91.6%. In the severest condition tested (230 °C), the solid still contained 2.1% xylan.

Table 3. Effect of the severity factor on the solid yield (% of dry mass of feedstock) and chemical composition (%, dry basis) of the solids obtained by hydrothermal treatment of extracted ladanifer residues

	Severity Factor, log R_o								
	2.01 (150°C)	2.58 (170°C)	2.89 (180°C)	3.07 (190°C)	3.36 (200°C)	3.51 (205°C)	3.65 (210°C)	3.98 (220°C)	4.25 (230°C)
Solid yield	88.9	85.9	79.5	72.1	70.1	65.7	61.3	60.1	60.9
Glucan	27.2	27.5	29.4	31.3	30.5	34.7	34.0	34.0	34.1
Xylan	16.4	16.6	14.5	12.2	10.8	9.1	6.2	3.6	2.1
Arabinan	2.0	1.4	1.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Acetyl groups	3.1	3.2	2.3	1.9	1.6	1.0	0.4	0.0	n.d.
Klason lignin	33.2	34.7	38.3	41.1	43.0	44.7	48.6	52.5	54.1
Ash	3.5	3.3	3.2	3.4	3.4	3.3	3.5	3.3	3.1
Protein and others (by difference)	14.6	13.3	11.3	10.1	10.7	7.2	7.3	6.6	6.6

n.d., not detected; values in parentheses indicate the reaction temperature

The hydrolysis of glucan was low, but still significant (solubilization, 12.9–26.6%). Nevertheless, as hemicellulose is removed at a higher rate, solid residues present an increased cellulose content, so that the pretreated biomass is enriched in cellulose, in a similar trend as previously reported for the autohydrolysis of other lignocellulosic materials (Carvalheiro et al., 2009; Bensah et al., 2015).

Lignin seems not to be extensively solubilized by the autohydrolysis (Fig.1c), and therefore, the solid residue showed an increase in Klason lignin content. Under the most severe conditions, the solid fraction contained 54.1% of lignin. However, as described above, phenolic compounds possibly derived from lignin also appear in hydrolysates. So that lignin solubilization was putatively counterbalanced by the formation of pseudo-lignin.

In order to characterize the processed solid and to clarify changes on lignin composition, Py-GC/MS was applied to the pretreated solid resulting from the hydrothermal treatment at log R_0 = 3.51, for which high XOS and glucan yields were obtained and also to untreated biomass (CLRext). Table 4 lists the main components identified in the chromatograms. The results obtained by the comparison of both pyrograms show that the hydrothermal treatment induced changes in the chemical structure of CLRext, namely with regard to the yields of some individual components, although most derivatives maintained similar contents in both untreated and pretreated samples. As shown in Table 4, autohydrolysis increased the content of the carbohydrate-

derived compounds from 70 to 76% (untreated *vs*. treated samples). This is in agreement with the wet chemical analysis, which also showed a relative increase of carbohydrates (glucan) in the hydrothermally pretreated solid (205 °C) (Tables 1 and 4). Levoglucosan is the main carbohydrate-derived pyrolysis product from cellulose (Patwardhan et al., 2009), and other studies showed its increase in pyrolyzed samples after hydrothermal treatment (Johnson et al., 2009; Mante et al., 2014; Zhurinsh et al., 2017). Levoglucosan yields resulting from hydrothermal treatments were attributed to the increase in the content of "active cellulose" (Johnson et al., 2009), to hydrolytic processes accompanied by cellulose activation and protonation of glucosidic linkages by organic acids (Zhurinsh et al., 2017), and also to a reduced amount of inorganics in the hydrothermally treated samples (Mante et al., 2014).

Table 4. Identification and quantification (as % of total area of the pyrogram) of the main pyrolysis products as a function of their origin in samples of extracted *C. ladanifer* distillery residues and hydrothermally pretreated at 205 °C (log R_0 = 3.51)

Peak nº	RT	Compound	Origin	CLRext	log R_0 = 3.51 (205°C)
1	5.82	2-oxo-propanal	C	3.9	3.7
2	8.14	Hydroxyacetaldehyde (HAA)	C	5.1	4.5
3	9.08	acetic acid	C	8.7	3.8
4	10.06	2-hydroxypropanone	C	1.0	0.8
9	12.86	3-hydroxypropanal	C	2.8	1.4
18	14.76#	furfural	C	1.9	1.2
28	19.53	not identified sugar	C	2.0	0.7
30	21.39	4-hydroxy-5,6-dihydro-2*H*-pyran-2-one	C	4.1	2.1
39	28.32	4-methylguaiacol	G	0.6	0.9
42	31.82	4-ethylguaiacol	G	0.05	n.d.
43	32.01	not identified sugar	C	2.0	2.1
47	33.76	1,5-anhydro-arabinofuranose	C	2.3	n.d.
48	34.15#	4-vinylguaiacol	G	1.2	1.4
50	35.90	5-hydroxymethylfurfural	C	0.7	0.5
51	36.38	syringol	S	0.4	0.8
54	38.68	2-hydroxymethyl-5-hydroxy-2,3-dihydro-4*H*-pyran-4-one	C	2.4	2.8
57	40.02	4-methylsyringol	S	0.6	1.0
56	39.66	similar to 1,5-Anhydro-arabinofuranose	C	2.5	2.3
64	45.11	4-vinylsyringol	S	0.8	1.2
70	49.20	1,6-anhydro-β-D-glucopyranose	C	22.0	42.7
71	49.82	*trans* 4-propenylsyringol	S	0.4	0.8
74	54.65	*trans* coniferaldehyde	G	0.6	n.d.
Total carbohydrate derived compounds[a]				70	76
Total lignin derived compounds[a]				8.4	10.4
Sum of S-lignin-derived units[a]				3.5	5.1
Sum of G-lignin-derived units[a]				4.0	4.2

Sum of H-lignin-derived units[a]	0.5	0.6
NDL[a]	0.4	0.5
S/G[a]	0.9	1.2
S:G:H[a]	1 : 1.1 : 0.1	1 : 0.8 : 0.1

Compounds overlapped; a Calculated based on all pyrolysis products identified and not only on the main peaks presented in this table
n.d., not detected; C, carbohydrate-derived products; G, derived from G-lignin units; S, derived from S-lignin units; H, derived from H-lignin units; NDL, lignin origin not identified

The main compounds that decreased after hydrothermal treatment were the following: hydroxyacetaldehyde (HAA) (peak 2), acetic acid (peak 3), 3-hydroxypropanal (peak 9), furfural (peak 18), 4-hydroxy-5,6-dihydro-2H-pyran-2-one (peak 30), 1,5-anhydro-arabinofuranose (peak 47), and 5-hydroxymethylfurfural (peak 50). HAA had a decrease of about 12% in relation to initial biomass. Johnson et al. (2009), using hot compressed water, verified that reaction selectivity of cellulose can foster depolymerization reactions, leading to formation of levoglucosan in detriment of fragmentation reactions, thereby resulting into the formation of HAA. Acetic acid decreased about 56% with the autohydrolysis. In general, acetic acid is formed from acetyl groups linked to the xylan backbone, and therefore, the removal of xylan by the hydrothermal treatment leads to its decrease in the pyrolysis products of the pretreated biomass as described above. As expected, a reduction of pentose-derived carbohydrates was promoted by the treatment: 4-hydroxy-5,6-dihydro-2H-pyran-2-one decreased by approximately 48%, while 1,5-anhydro-arabinofuranose was totally removed from the pyrolysis products from the pretreated biomass. Furan derivatives, e.g., furfural and HMF, decreased 37% and 57%, respectively, after hydrothermal treatment.

Autohydrolysis also led to an increase of about 24% in the total content of lignin-derived compounds from syringyl (S), guaiacyl (G), and p-hydroxyphenyl (H) units (Table 4). Two guaiacol components (4-ethylguaiacol, peak 42 and trans-coniferaldehyde, peak 74) were not conserved in the pyrolysis products after the pretreatment. The main compound identified was 4-vinylguaiacol (peak 48), followed by 4-vinylsyringol (peak 64), 4-methylsyringol (peak 57), 4-methylguaiacol (peak 39), syringol (peak 51), and trans-4-propenylsyringol (peak 71). These lignin-derived compounds increased in the pretreated biomass.

The change in lignin composition with the hydrothermal treatment may be followed by comparing the S/G ratio of CLRext and of CLRext after the hydrothermal treatment (0.9 and 1.2, respectively) showing a decrease of guaiacyl units and an increase of syringil units, what is compatible to the proposal that lignin is partly solubilized and reacts to form pseudo-lignin. The hydrothermally treated biomass may therefore show a better delignification in comparison with the untreated biomass since S units react more easily with alkaline reactants. This may favor the subsequent use of this pretreated biomass in the biorefinery framework.

4. Conclusions

Cistus ladanifer residues from commercial essential oil extraction facilities were subject to sequential extractives removal by ethanol and water yielding 40 g/100 g of CLR of extractives and a solid residue (CLRext) containing mainly polysaccharides (51%) and lignin (32%). The extractives contain potential bioactive compounds (mainly gallic acid and flavonoids) that can potentially be used in a wide range of added-value applications. Furthermore, the removal of these extractives is an important step in the fractionation process as it enables to obtain alternative products from this residual biomass but also because it improved the oligosaccharide yields attained after hydrothermal processing. The highest concentration of oligosaccharides (~ 24 g/L) was similar from log R_0 3.07–3.51 (190–205 °C) and corresponds to a yield of 15 g oligosaccharides/100 g feedstock. Under these conditions, the production of monosaccharides and aliphatic acids also occurred reaching 4.5 g/L and 1.8 g/L, respectively. Thus, the hydrothermal processing of extractive free (CLRext) residues led also to a decrease of both monosaccharide and inhibitor compounds which is and advantage of these hydrolysates for bioactive (e.g., prebiotic) or fermentation applications. Besides this, the autohydrolysis affected the monomeric composition of the residual lignin, causing an increase of the S/G ratio. The solid cellulose- and lignin-enriched fraction produced shows a composition potentially favorable for other applications.

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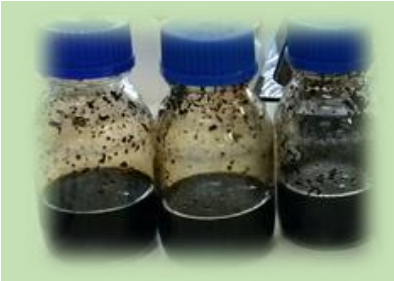
CAPÍTULO VI



Delignification of *Cistus ladanifer* biomass by organosolv and alkaline processes and products characterization

A informação constante neste capítulo está baseada na publicação:

Júnia Alves-Ferreira, Ana Lourenço, Francisca Morgado, Luís C. Duarte, Luísa Roseiro, Maria C. Fernandes, Helena Pereira, Florbela Carvalheiro (2019). Delignification of *Cistus ladanifer* biomass by organosolv and alkaline processes and products characterization. (submetido)

Abstract

Residues of *Cistus ladanifer*, a native species of the Mediterranean region, obtained after steam distillation for essential oil production were evaluated to produce cellulose enriched solids and as a source of added-value lignin-derived compounds. Extracted and hydrothermally pretreated biomass was delignified by two organosolv processes, ethanol/water mixtures (EO) and alkaline-catalysed glycerol (AGO), and by an alkaline (sodium hydroxide) process (ASP) under different reaction conditions, targeting the recovery of lignin-derived phenolic compounds from the liquid stream and the production of cellulose-rich solids. The phenolic composition of soluble lignin was determined by capillary zone electrophoresis and the lignin monomeric composition by Py-GS/MS. The enzymatic saccharification of the delignified solids was also evaluated. ASP (4% NaOH, 2h) lead to both the highest delignification and enzymatic saccharification (87% and 79%, respectively). A delignification of ~76% and enzymatic hydrolysis yields of 72% were obtained for AGO (4% NaOH) while EO processes led to lower delignification (maximum lignin removal 30%).

The residual lignins in the delignified solids were enriched in G- and H-units, with S-units being preferentially removed. The main phenolics in the ASP and AGO liquors were vanillic acid and epicatechin, while gallic acid was the main phenolic in the EO liquors. The results showed that *C. ladanifer* residues can be a biomass source for the production of lignin-derivatives and glucan-rich solids to be further used in bioconversion processes.

Keywords: Biorefinery, Enzymatic hydrolysis, Glucan-enriched solids, Lignin-derived products, Analytical pyrolysis; Rock-rose

1. Introduction

Lignin is an important source of compounds with functional activities and emerging applications in cosmetic, pharmaceutical and food industries. Compared to cellulose, the upgrading of lignin has deserved much less attention due to its recalcitrance, as well as its chemical and structural complexity. Although lignin is rich in phenolic and aliphatic hydroxyl groups, which are functional groups of interest for chemical modifications and reactions, it is often used only as filler or additive since separation and fragmentation processes are some of the hindrances for its utilization for production of chemicals (Laurichesse & Avérous, 2014). However, there has been a renewed interest in the study of lignin extraction processes to obtain added value products (Toledano et al., 2010). These products can be grouped into three categories: biofuels, macromolecules and monomeric aromatic compounds (Holladay et al., 2007). The lignins can be incorporated into polymeric materials, including conducting polymers, polyurethanes and thermoplastic sealants (Varanasi et al., 2012). They can also be used for the production of phenolic resins, binders and adhesives (Holladay et al., 2007; Stewart, 2008). However, the interest in the production of monomeric phenolic compounds, such as vanillin, ferulic acid, coumaric acid, p-hydroxybenzoic acid, vanillic acid, syringic acid, syringaldehyde and p-hydroxybenzaldehyde, for example, is having increased interest. In fact, the development of new technologies for the production of lignin-based chemicals can lead to much higher value market opportunities than petrochemicals (Berlin & Balakshin, 2014). The market potential for high added-value products such as vanillin, phenol, BTX (benzene, toluene and xylene) and carbon fibers, for example, is over $130 billion, and in 2020 this potential is expected to reach $208 billion (Smolarski, 2012).

The lignin source, as well as the extraction processes used to break down the lignin macromolecule into fragments of lower molecular mass, have an important influence on the physicochemical properties of the resulting products (Erdocia et al., 2014). The most used delignification processes involve the action of alkalis that although mainly reacting with lignin also affect the hemicelluloses. The most used agents are alkali metal hydroxides (sodium, potassium or calcium) and one of the most known examples is the Kraft process that uses sodium sulphide and sodium hydroxide that is currently used in pulp and paper production (Sridach, 2010; Gosselink, 2011). It has the advantage that it may be performed at low temperatures, although the quality of lignin is rather low. These processes produce, at present, the highest volume of lignin but are slight specific and required significant operations of wastewater treatment.

Organosolv processes are based on the utilization of aqueous mixtures of organic solvents such as acetone, ethanol, methanol, glycerol, formic acid and acetic acid (Demirbaş, 2004; Xu et al., 2006; Sun & Chen 2008; Erdocia et al., 2014; Cybulska et al., 2015). These are interesting delignification alternatives since they allow to obtain lignin with low ash content, higher purity (due to lower content of carbohydrates) and, in general, with low molecular weight and higher hydrophobicity (Moniz et al., 2018). In addition, the organosolv treatments lead to a liquid phase containing both hemicellulose and lignin derived products that are free from sulphur (Xu et al., 2006). Organosolv processes can also be used in combination with other catalysts, including acids, leading to lignin dissolution and hemicellulose hydrolysis (Bozell et al., 2011). The overall economy of these processes depends on solvent recycling, although solvents such as ethanol and acetone have the advantage of easy recovery by distillation and are themselves biorefinery products (Carvalheiro et al., 2008).

Besides the recovery of lignin for conversion into valuable products, these treatments may also produce cellulose-rich solids with enhanced enzymatic hydrolysis rate and yield (Brodeur et al., 2011).

The biomass used in this study was *Cistus ladanifer*, a native specie in the Mediterranean region also used for essential oil production. The remained residues obtained after distillation present a favourable chemical composition to be used as a biorefinery feedstock (Alves-Ferreira et al., 2017). The combination of extraction, hydrothermal processing (autohydrolysis) and delignification treatments are important processing routes for the full use of this biomass. Thus, the solids remaining from previously extraction and optimised hydrothermal processing for hemicellulose fractionation were subjected to delignification treatments using two mild organosolv processes: ethanol/water mixtures and alkaline-catalysed glycerol. For comparative purposes, an aqueous sodium hydroxide process was tested and the processes were also applied to the only extracted biomass.

This work aims to study the operational conditions for the delignification of extracted (CLRext) and extracted and pretreated solids (CLRtreat) of *Cistus ladanifer* residues using different delignification processes in order to produce cellulose rich solids and maximize the recovery of the lignin-derived phenolic compounds in the liquid fraction. Lignins and remaining solids were characterised and the influence of the extraction methods on their physicochemical properties was evaluated.

2. Material and methods

2.1. Raw material

The samples used in this work were *Cistus ladanifer* residues (CLR) obtained after essential oil distillation, also after extraction with ethanol and water (CLRext) and pretreated by an autohydrolysis (hydrothermal) process (CLRtreat) that was previously optimized (Alves-Ferreira et al., 2019). The hydrothermally pretreated solids were produced in sufficient amount and carefully homogenized to be used for the delignification studies. The average chemical composition of the CLRext used in this work was 29% glucan, 17% xylan, 4% arabinan, 3% acetyl groups and 30% Klason lignin and CLRtreat contained 35% glucan, 9% xylan, 1.4% acetyl groups and 45.8% lignin

2.2. Delignification treatments

2.2.1. Aqueous sodium hydroxide process (ASP)

CLRext and CLRtreat were used as feedstock for an aqueous sodium hydroxide process (ASP). The treatment was carried out in an autoclave at 130 ºC during 60 or 120 min. Each treatment used 10 g of solids (dry weight) and sodium hydroxide (NaOH) solutions at 2% or 4% (w/v) with a solid:liquid ratio of 1:10 (w/w). At the end of the reaction, the flasks were cooled down to room temperature and the liquid and solid fractions were separated by filtration (Quantitative Filter-Lab nr 1235 filter paper). The liquor was stored at 4 °C for further analysis and the solid phase was washed with 1L of hot distilled water, dried for 48 h at 45 ºC and kept for another 48 h at room temperature before milling and storage until analysis.

2.2.2. Alkaline-catalyzed glycerol organosolv (AGO)

Alkaline glycerol organosolv (AGO) was performed similarly to ASP. The delignification was carried out at 130 ºC during 60 min and each treatment used CLRtreat and 50:50 water/glycerol (w/w) solution containing 2, 4 or 8 g of NaOH as catalyst, in a solid:liquid ratio of 1:10 (w/w). After separation the solid phases were washed with 1% NaOH solution (w/v) followed by 2L of hot distilled water to remove possible adsorbed lignin.

2.2.3. Ethanol organosolv process (EO)

The ethanol organosolv process (EO) used CLRtreat solids. The process was performed in a 600 mL stainless-steel reactor (Parr Instruments Co, USA) using 50:50 ethanol/water mixtures and a 6:1 liquid-to-solid ratio (w/w). The temperature, agitation and pressure were controlled by a Parr PID controller (model 4842). The reaction was carried out at non-isothermal conditions in a temperature range of 170 to 220 ºC. Upon reaching the desired temperature, the reactor was cooled down by water circulating through a serpentine coil and introduced in a cold-water bath. The solid and liquid phases were separated by filtration and washed with 1L ethanol/water solution and dried as described in 2.2.1.

The diagram of sequential fractionation methods (extraction, autohydrolysis and delignification) applied to this biomass is illustrated in Fig. 1.

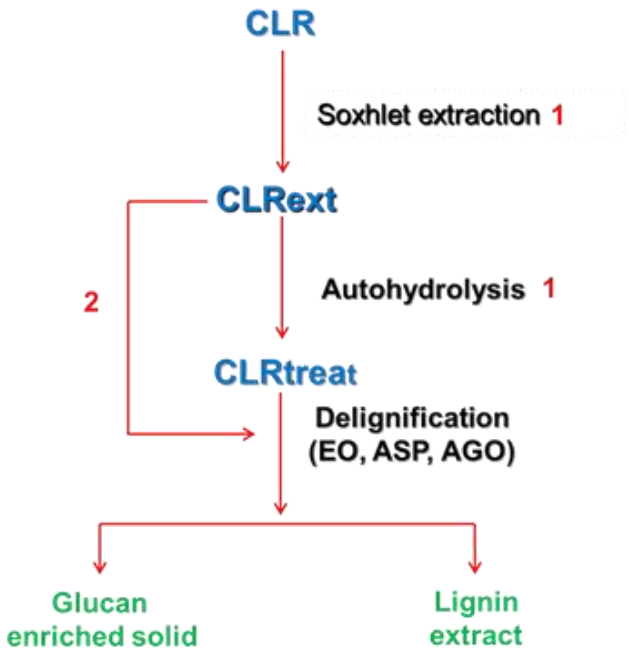


Fig. 1 The scheme of sequential fractionation methods applied to *Cistus ladanifer* residue

[1] Alves-Ferreira et al., 2019

[2] Aqueous soda delignification (only)

CLR – *Cistus ladanifer* residues

CLRext – *Cistus ladanifer* residues extracted

CLRtreat – *Cistus ladanifer* residues extracted and hydrothermally treated

2.3. Lignin precipitation

Lignin was separated from the ASP and AGO liquors by acid precipitation with 72% H_2SO_4 to pH 2. The lignins precipitated from the ASP and AGO liquors were filtered, washed with hot distilled water and dried at 45 °C for 24 h. For the precipitation of the EO lignin, the liquor was diluted with distilled water (3:1), centrifuged at 5000 rpm for 10 min and dried at 45 °C for 24 h.

2.4. Analytical methods

2.4.1. Quantification of carbohydrates and lignin

The solids obtained after each delignification process were subjected to quantitative acid hydrolysis (QAH) according to the conditions used in a previous work (Alves-Ferreira et al. 2017). The liquors resulting from QAH were analyzed for glucose, xylose, arabinose and acetic acid by HPLC (Agilent, Waldbronn, Germany) as described by Moniz et al. (2013). The solid residue QAH was corrected for ash and considered as Klason lignin. The moisture content was determined by oven-drying at 100 °C to constant weight and the ash content was determined at 550 ºC using NREL/TP-510-42622 protocol (Sluiter et al., 2008).

2.5. Chemical characterization of lignin and lignin-derived compound

2.5.1. Phenolic profile by Capillary Zone Electrophoresis (CZE)

The lignin-derived products contained in the delignification liquors were analyzed by CZE (Agilent System, Waldbronn, Germany), equipped with a diode-array detector (DAD) and interfaced with a ChemStation data software under the operating conditions previously reported in Alves-Ferreira et al (2019). Detection was performed at 200 and 375 nm, and phenolic compounds were identified by electrophoretic comparisons (migration times and UV spectra) using authentic standards.

Total phenolic compounds were determined by the Folin–Ciocalteu colorimetric method using a microplate spectrophotometer (Thermo Scientific, USA) as detailed elsewhere (Alves-Ferreira et al., 2019) and expressed as g of gallic acid equivalents per liter of liquor (gGAE/L).

2.5.2. Pyrolysis experiments

Py-GC/MS was applied in order to study and compare the modifications on delignified solids and in the lignin isolated from the liquors. The solids analyzed were those corresponding to the conditions with the highest delignification obtained for each treatment (EO5, AGO3, ASP8) and the CLRtreat, for comparison purposes. The lignins in the liquors correspond to precipitated EO5, AGO3, ASP7 and ASP8 liquors. The samples were pyrolyzed at 550 ºC for 10 s in a 5150 CDS apparatus (CDS Analytical, USA) linked to an Agilent GC 7890B coupled to a mass detector system 5977B using electron impact mode (EI at 70 eV) according to the operating conditions described before (Alves-Ferreira et al., 2019). The pyrolysis products were identified by comparison with computer libraries (Wiley, NIST2014) and with literature (Faix et al., 1991; Ralph & Hatfield 1991). The percentage of each compound was calculated based on total area of the chromatogram. The percentage of hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin-derived products were separately summed. The S/G ratio and the S:G:H relation were calculated.

2.6. Enzymatic hydrolysis

The enzymatic digestibility of cellulose of the delignified solids was evaluated using reference protocols (LAP-NREL/TP-510-42629) (Selig et al., 2008). The dried solid fraction from the delignification process was digested in 5 mL of sodium citrate 0.1 M pH 4.8 buffer solution, 100 µL solution of sodium azide (2% w/v) and then supplemented with 60 FPU/g cellulose of Celluclast 1.5L and 64 pNPGU/g cellulose of Novozyme 188 (Novozymes, Denmark). The volume was adjusted with distilled water to 10 mL considering that the biomass has a density of 1. Incubation was carried out 150 rpm in an orbital incubator at 50 °C, for 72 h, in triplicate. Monomeric composition of polysaccharides was assessed by HPLC (RI detector, UV/Vis DAD, column Bio-Rad HPX-87H) as described in Alves-Ferreira et al. (2019). The enzymatic digestibility was determined by the ratio of digested cellulose to the initial cellulose loaded.

Filter Paper Activity (Celluclast 1.5L) and β-glucosidase activity (Novozyme 188) were determined as 61 FPU/mL and 481 IU/mL, according to the procedures of Ghose (1987) and Berghem et al. (1975), respectively.

3. Results and discussion

3.1. Chemical composition of the delignified solids and delignification yield

The experimental conditions of the delignification processes and the chemical composition of the remaining solids, delignification yield and solid yield obtained after treatment are shown in Table 1.

The ethanol organosolv process solubilized less lignin when compared to the other delignification methods, resulting in a solid residue still with high lignin content. The delignification obtained ranged from 13% to 28.9%, increasing with temperature increase up to 210 °C but dropping at 220 °C. Wildschut et al. (2013) also observed that delignification tend to increase with the increase of reaction temperature. The results obtained here are lower than those reported for ethanol-water delignification of other feedstocks e.g.: palm fronds, 80% ethanol at 200 °C (Cybulska et al., 2015); sugarcane bagasse, 30% ethanol at 195 ºC (Mesa et al., 2011). It is also important to note that in general a catalyst, e.g. sulphuric acid, is also added. This was the case of the experiments carried out by (Wildschut et al., 2013; Chen et al., 2015) where 30 mM of sulphuric acid was used for the delignification of wheat straw with 60% ethanol at 190 °C. The hemicellulosic fraction was only slightly affected by EO, except at 220 °C, where a major decrease was observed (corresponding to a solubilization around 50%). Between 170 ºC and 210 ºC there was a slight solubilization of the xylan from 13% to 28%, while glucan mainly remained in the solid phase (maximum solubilization 7%). Since the ethanol organosolv treatment was not very effective for lignin removal, it was not possible to obtain solids with high glucan content (it ranged from 35.2% to 44.3%).

In order to try to improve the former results, sulphuric acid (50 mM) was also tested as a catalyst at 220 °C in the organosolv process. However, even under these conditions the performance of ethanol organosolv process for lignin solubilization was not improved but xylan removal increased to reach 78% of solubilization. Ethanol organosolv was also performed under isothermal conditions, but the lignin yields were even less satisfactory than those using non-isothermal conditions (data not shown).

The alkaline-catalyzed glycerol organosolv using NaOH as catalyst (1%, 2% and 4% (w/w)), for 60 min, at a mild temperature of 130 °C exhibited a greater delignifcation degree in comparison to the ethanol organosolv assays. The highest lignin removal was obtained for 4% NaOH concentration (AGO3), reaching a delignification yield of 76.3% and resulting in a solid containing 67.7% glucan, 4.4% xylan and 18.4% residual lignin. AGO1 and AGO2 produced a delignification yield of 37.4% and 69.1%, respectively. The

maximum delignification yield reached out for AGO was higher than the obtained by Sun et al. (2015) for wheat straw, using 70% industrial glycerol at 220 °C for 3 h, and lower to that showed by Hundt et al. (2013) for beech wood, using 97% glycerol and 8% KOH as a catalyst, at 190 °C, for 15 min. Novo et al. (2011) reported delignification yields between 21–82% for sugarcane bagasse, using 80% glycerol under different conditions of time and temperature. The solid yield decreased with the NaOH concentration, achieving 73.4%, 61.0% and 60.6% for AGO1, AGO2, and AGO3, respectively. The amount of xylan solubilized also increased with the NaOH concentration (maximum solubilization 68%), while acetyl groups were completely removed from the solid residue at all the conditions. Glucan was almost not affected by AGO: a solubilization of 12% occurred for AGO1 (using 2% NaOH) and there was not any degradation of glucan for the other conditions. Other studies with glycerol organosolv extraction methods also demonstrated high preservation of cellulose in the substrate (Sun et al., 2015; Meighan et al., 2017).

For comparative purposes, an aqueous soda process (ASP) was also tested with two NaOH concentrations (2% and 4% (w/v)) and two-time periods (1 and 2 h) after reaching a temperature of 130 ºC. This process was also applied to extracted biomass (CLRext) and hydrothermally treated biomass (CLRtreat). ASP showed an important influence in the delignification of CLRext and CLRtreat, being the most efficient method tested in this work for lignin extraction. The delignification corresponded to percentages from 68% to 78% for CLRext and from 73% to 87% for CLRtreat. Therefore, CLRtreat allowed the maximum lignin removal ca. 11.5% superior to the maximum removal obtained with CLRext. The results of delignification obtained for CLRext were similar taking into account the different reaction times (ASP1 *vs*. ASP2 and ASP3 *vs*. ASP4). For the CLRtreat, the delignification degree only improved 3.3% with the increase of reaction time (ASP5 vs. ASP6 and ASP7 vs. ASP8). These results suggest that NaOH concentration may have an influence on lignin removal, while the reaction time seems not to have an important effect. Studies done by Wu et al. (2018) demonstrated that the increase of the alkaline concentration is important to improve the delignification degree of wheat straw. The hydrolytic capacity of NaOH is well known since it cleaves the ether and ester linkages in the lignin-carbohydrate complexes, and the ester and carbon-to-carbon bonds in lignin (Fengel & Wegner, 1984). Different concentrations of NaOH solutions (0.3–10%) under temperatures ranging from 70 °C to 160 °C and 1–3 h reaction times led from moderate to high delignification yields (56–97%) in different lignocellulosic biomass (Huang et al., 2015; Nagula & Pandit, 2016; Wang et al., 2017).

Table 1. Experimental conditions for the delignification of *Cistus ladanifer* residues before (CLRext) and after hydrothermal treatment (CLRtreat) using three delignification processes - ethanol organosolv (EO), alkali-catalyzed glycerol (AGO) and soda process (ASP) - and results regarding delignification yield (% of the initial o.d. material), solid yield (% of the delignified solid), contents of Klason lignin, glucan and xylan (% of the delignified solid)

Treatments								**Delignification**				
Process	**Nr**	**Reagent**	**Feedstock**	**Reactor**	**Temperature (°C)**	**Time (h)**	**pH**	**Delignification yield (%)**	**Solid Yield**	**Klason Lignin (%)**	**Glucan (%)**	**Xylan (%)**
EO	1	50% ethanol	CLRtreat	Parr	170	NI	4.4	13.3	88.2	46.0	35.2	8.8
EO	2	50% ethanol	CLRtreat	Parr	180	NI	4.4	17.0	86.0	45.2	36.6	9.2
EO	3	50% ethanol	CLRtreat	Parr	190	NI	4.2	20.0	85.3	43.9	39.8	8.7
EO	4	50% ethanol	CLRtreat	Parr	200	NI	4.2	22.4	81.1	44.7	38.9	8.3
EO	5	50% ethanol	CLRtreat	Parr	210	NI	4.2	28.9	79.9	41.7	44.3	8.3
EO	6	50% ethanol	CLRtreat	Parr	220	NI	4.1	21.6	80.6	46.5	40.1	5.6
AGO	1	50% glycerol + 1% NaOH	CLRtreat	AC	130	1	8.0	37.4	73.4	40.2	42.0	6.4
AGO	2	50% glycerol + 2% NaOH	CLRtreat	AC	130	1	10.5	69.1	61.0	23.9	61.0	5.1
AGO	3	50% glycerol + 4% NaOH	CLRtreat	AC	130	1	11.8	76.3	60.6	18.4	67.7	4.38
ASP	1	2% NaOH	CLRext	AC	130	1	9.9	68.8	49.0	20.2	45.7	20.0
ASP	2	2% NaOH	CLRext	AC	130	2	10.4	68.7	48.7	19.1	46.0	21.2
ASP	3	4% NaOH	CLRext	AC	130	1	12.9	77.7	39.8	16.7	51.9	21.4
ASP	4	4% NaOH	CLRext	AC	130	2	12.8	78.3	39.7	16.2	52.8	21.5
ASP	5	2% NaOH	CLRtreat	AC	130	1	9.7	72.8	49.3	24.1	63.5	8.4
ASP	6	2% NaOH	CLRtreat	AC	130	2	10.5	75.2	46.8	23.1	64.4	7.7
ASP	7	4% NaOH	CLRtreat	AC	130	1	12.9	83.9	43.0	16.3	74.0	7.6
ASP	8	4% NaOH	CLRtreat	AC	130	2	12.8	86.7	39.8	14.6	76.8	7.2

NI, Non-isothermal conditions; AC, Autoclave

Contrarily to other treatments, ASP had a slightly higher effect in glucan solubilization than the organosolv processes. This was mainly evident for the untreated biomass (about 14.9–18.2% for CLRtreat vs. 24.3–30.2% for CLRext). For CLRext glucan degradation increased with alkali load. However, this effect was not observed for CLRtreat. This is an advantage as CLRtreat is a feedstock that is the by-product of *C. ladanifer* distillation (CLR), of CLR extraction (CLRext) and of CLRext hydrothermal treatments to produced oligosaccharides and it is still very interesting to produce cellulose-enriched solids and lignin extracts. The alkaline treatment also had an important effect on the xylan fraction, with solubilizations of approximately 50% and 71% for CLRext (ASP4) and CLRtreat (ASP8), respectively. Similar to what occurred in AGO, acetyl groups were also fully solubilized at all conditions (data not shown). Arabinan was completely removed from CLRtreat by hydrothermal pretreatment, but CLRext still contained 4% arabinan. An almost complete solubilization of arabinan from CLRext was achieved in any of the conditions (92.5–94% of solubilization) (data not shown). The ASP process displayed the lowest solid yields among all the treatments (from 39.7% to 49.3%) due to the higher lignin and carbohydrate hydrolysis.

According to these results, EO was the less effective delignification process, while the highest delignification and glucan yields were observed in the ASP followed by AGO, suggesting the efficiency of alkaline process for delignification of *C. ladanifer* in order to obtain a feedstock suitable for obtaining glucose solutions and lignin-derived phenolics.

The pH of the liquors from the alkaline glycerol and soda treatments was 11.8 and 12.9, respectively, with higher pH values for the higher soda concentrations (Table 1). Ethanol organosolv liquors exhibited similar values (4.1–4.4), with a slight pH decrease with increasing temperature. The values obtained are lower that the reported by Wildschut et al. (2013) in ethanol organosolv liquors (4.2 to 5.9).

3.1. Enzymatic hydrolysis

The results of the enzymatic digestibility of the solid fractions remained after delignification are presented in Fig 2. The extraction of lignin using chemicals, besides the rupture of lignin, leads to a swelling of the biomass, as well as to the increase in internal surface area and a better access of hydrolytic enzymes (Agbor et al., 2011).

EO treatment induced an increase in digestibility with the increase of reaction temperature. Organosolv performed at high temperatures may cause a higher rupture of the cell wall structure (Chen, et al., 2015) and therefore a better enzymatic access. However, the glucose yields obtained with EO solids were only about 39.3% to 49.3%. These results are in

agreement with the lower delignification of CLRtreat obtained with these processes and contrast with the previous reported by Wildschut et al., 2013. These authors obtained a maximum enzymatic digestibility of 86% and 89% for wheat straw using aqueous ethanol without and with catalyst, respectively. Jang et al. (2016) also reported a digestibility over 80% for ethanol organosolv of *Liriodendron tulipifera*.

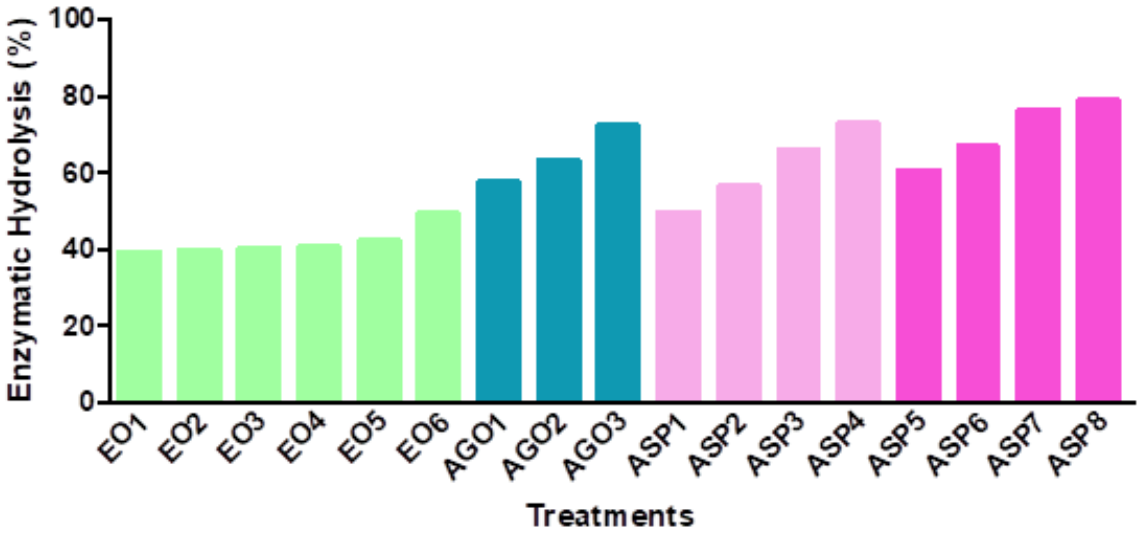


Fig. 2 Results of the glucan enzymatic hydrolysis (% of hydrolyzed glucan from delignified solid): ethanol organosolv (EO), alkali-catalyzed glycerol (AGO) and soda process (ASP)

The results obtained for AGO and ASP solids showed a significant increase in the hydrolysis compared to the EO. Thus, values between 57.5–72.3% of cellulose digestibility were observed for AGO processed solids. These results are lower than those obtained for wheat straw (wet substrate) using crude and industrial glycerol (Sun and Chen, 2008; Sun et al., 2015). Also, beech wood alkaline glycerol delignification (Hundt et al., 2013) and sugarcane glycerol/ sulphuric acid delignification (Martin et al., 2011) produced higher saccharification yields. CLRext (ASP1 to ASP4) showed enzymatic hydrolysis yields from 49.7% to 72.9% which are slightly below those obtained for CLRtreat (from 60.7% to 78.8%). Huang et al. (2015) achieved 81% enzymatic hydrolysis of a sample pretreated by 10% NaOH at 160 °C and Wang et al. (2017) indicated a hydrolysis efficiency of 79.2% for *Sophora flavescens* residues pretreated with 1.2% NaOH at 120 °C, for 2 h. Cellulose digestibility is increased with removal of barriers such as lignin, xylan and acetyl groups (Pan et al., 2006a; Zhao et al., 2012) and this could be observed in this work.

Therefore, the results showed that ASP and AGO are quite more effective than EO for fractionation of cellulose and lignin from *C. ladanifer* distillery residues as well as for the subsequent enzymatic hydrolysis of cellulose.

3.1. Phenolic composition of lignin liquors

The liquid fractions resulting from delignification were characterized for total phenolics concentration (expressed as gallic acid equivalents) and by CZE for their phenolic profile. In general, the phenolic compounds yield increased with the delignification yield. However, this was not always observed when analysing the samples individually. Ethanol extraction yielded lower total phenolic content (0.9–1.4 g/L), while AGO and ASP processes resulted in phenolic concentrations between 6.2 and 10.8 g/L (data not showed). Concentrations from 4.8 to 6 g/L of total phenolics were found for organosolv liquors from rice straw (Moniz et al., 2018). Phenolic compounds have shown interesting bioactivities, in particular, strong antioxidant potential. Thus, the antioxidant activity of lignins has suggested new applications of this polymer in cosmetics and pharmaceuticals industries (Pan et al., 2006b; Ugartondo et al., 2008).

Fig. 3 shows an example of the phenolic profile obtained by CZE where the lignin-derived non-volatile substances of EO6, AGO3 and ASP8 samples are shown. The electropherograms show the complexity of the liquor matrix, especially for AGO3 and ASP8, which were very viscous and with a great content of lignin. Gallic acid and vanillic acid are the phenolic acids identified in the ethanol liquor (Fig. 3A). These compounds were previously found in *C. ladanifer* extracts (Fernández-Arroyo et al., 2009; Barrajón-Catalán et al., 2010; Barros et al., 2010; Alves-Ferreira et al., 2019). Benzoic aldehydes such as vanillin and syringaldehyde could also be identified in the same sample at 375 nm (data not shown). In fact, vanillic acid was identified in all the samples, and especially in AGO3 and ASP8 this compound showed the most prominent peaks (Fig. 3B and 3C). Vanillin or, its oxidized form, vanillic acid, are known to exhibit potential bio-activities as antibacterial (Govindasami et al., 2011), inhibitory effects on initiation of hepatocarcinogenesis (Tsuda et al., 1994), protective effect on cisplatin-induced renal injury (Sindhu et al., 2015). In addition, vanillin is widely used as flavouring agent in food products and aromatic additives and its global market is expected to reach USD 724.5 million by 2025 (Grand View Research, 2017). The compounds 3-phenylphenol and epicatechin were found in AGO3 and ASP8 with a very good matching (Fig. 3B and 3C). Several studies reported that (−)-epicatechin, a flavan-3-ol, may contribute to prevention of cardiovascular diseases and metabolic disorders, and demonstrated blood pressure-lowering capacity (Bernatova, 2018). Other studies indicated pharmacological benefits of epicatechin such as inhibitory effects against human breast cancer cells (Nagarajan et al., 2008), insulin-like activity (Rizvi & Zaid, 2001) or parasitic activity (Zahir et al., 2012).

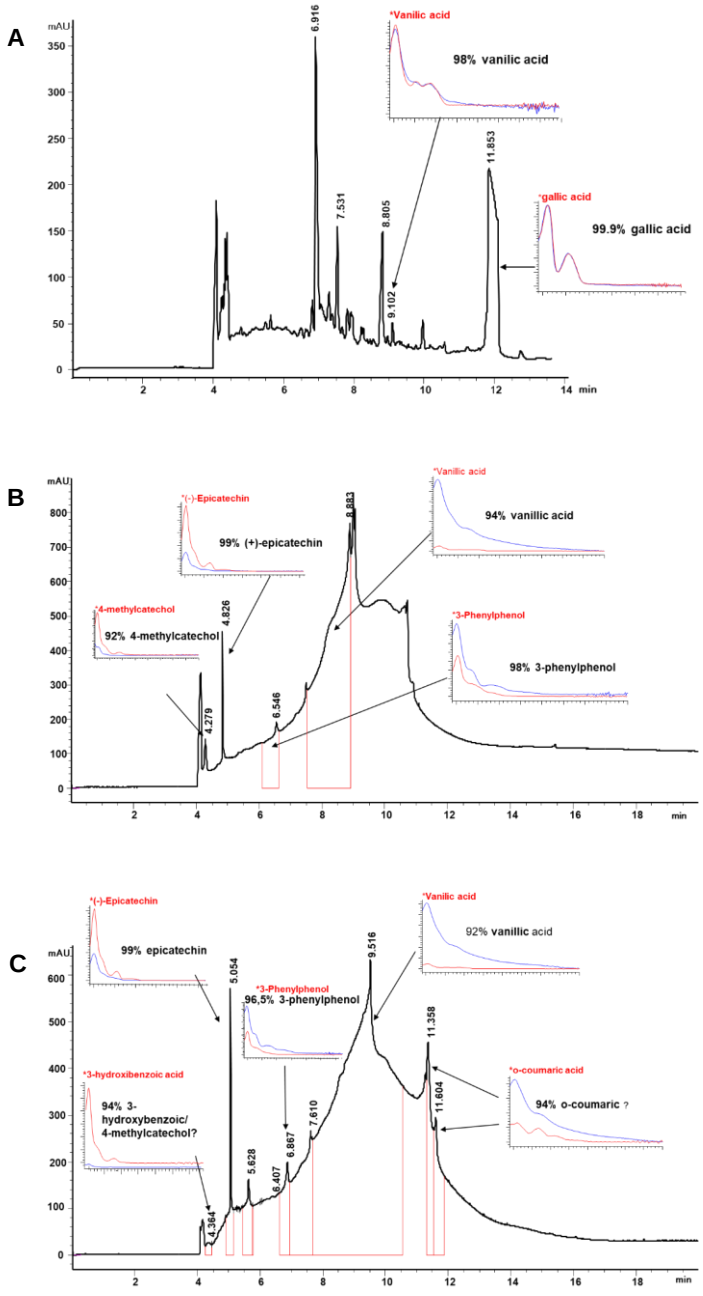


Fig. 3 Electropherogram (200 nm) showing the phenolic profile of the lignin products obtained after different delignification processes of treated Cistus ladanifer residues (CLRext). A) ethanol organosolv lignin (EO6 sample); B) alkaline glycerol organosolv lignin (AGO3 sample); C) soda lignin (ASP8 sample). The % matching was obtained by comparison with authentic standards run under the same conditions as the samples. See text for CZE conditions.

Other compounds such as coumaric acid, 3-hydroxybenzoic acid and 4-methylcatechol were detected in ASP8 and the later was identified also in AGO3. However, due to the matrix complexity and overlapping peaks, these compounds were not identified with a high degree of certainty. Moniz et al. (2018) also found coumaric acid in rice organosolv liquor. The biological activity of these phenolic compounds has been

demonstrated in numerous studies: 4-methylcatechol as an antimelanoma agent (Morita et al., 2003; Payton et al., 2011); coumaric acid as a strong antimicrobial (Maddox, et al., 2010; Liu et al., 2018) and antioxidant (Ou et al., 2009).

Therefore, the potential bioactivity of lignins supports their promising application in the pharmaceutical, cosmetic and food industries.

3.2. Characterization of delignified solids and isolated lignins by Py-GC/MS *pyrolysis*

The hydrotermally treated feedstock (CLRtreat), the delignified samples (EO5, AGO3, ASP8) and the isolated lignin from the liquors (EO5, AGO3, ASP7 and ASP8) were characterized by Py-GC/MS. Pyrolysis is a powerful methodology to characterise the monomeric composition of lignin in biomass (Lourenço and Pereira, 2018). Figures 4 and 5 show the pyrograms of the delignified solids and of the isolated lignins, respectively, and identify the numbers of the main peaks. Table 2 shows the identification, quantification and origin of the pyrolysis products.

The pyrograms of the solids remaining after delignification show a strong reduction of the lignin-derived products when compared to the lignin pyrograms (Fig. 4 and 5). In fact, the samples recovered from the liquor presented more lignin-derived pyrolysis products (from 49% to 78% of the total area) when compared to the delignified solids (from 7.7% to 11.5% of the total area). The proportion of S, G and H units, and of the S/G and S/G/H ratios were calculated from the total monomeric phenols identified in the pyrograms (Table 2).

CLRtreat has a S:G:H monomeric composition of 1:0.8:0.1, and a S/G ratio of 1.2. A G/S ratio of 0.60 for xylem and 1.29 for pith of *C. ladanifer* were determined in other studies (Micco & Aronne, 2007).

The lignin composition remaining in the delignified solids was changed after all the treatments. S-lignin represented 2.4% of pyrolysis products of the ASP8 sample, 3.7% of AGO3 and 4.2% of EO5 *vs.* 5.1% of the undelignified biomass. Compounds such as 4-methylsyringol (peak 73) and 4-vinylsyringol (peak 80) were preferentially attacked by the alkaline treatments, contributing to a more pronounced decrease of the S-units in the delignified solids of AGO and ASP than of EO process. This behaviour is consistent with the higher reactivity of syringyl lignin in delignification processes (Tsutsumi et al., 1995). Otherwise, there was an increase of G and H-type units in the delignified solids when compared to CLRtreat (respectively, from 4.2% to 4.7–5.6% and from 0.6% to 1.1–1.5%). The increase of G and H units and the decrease of S-units during delignification of *Eucalyptus globulus* was also reported by Lourenço et al. (2013a).

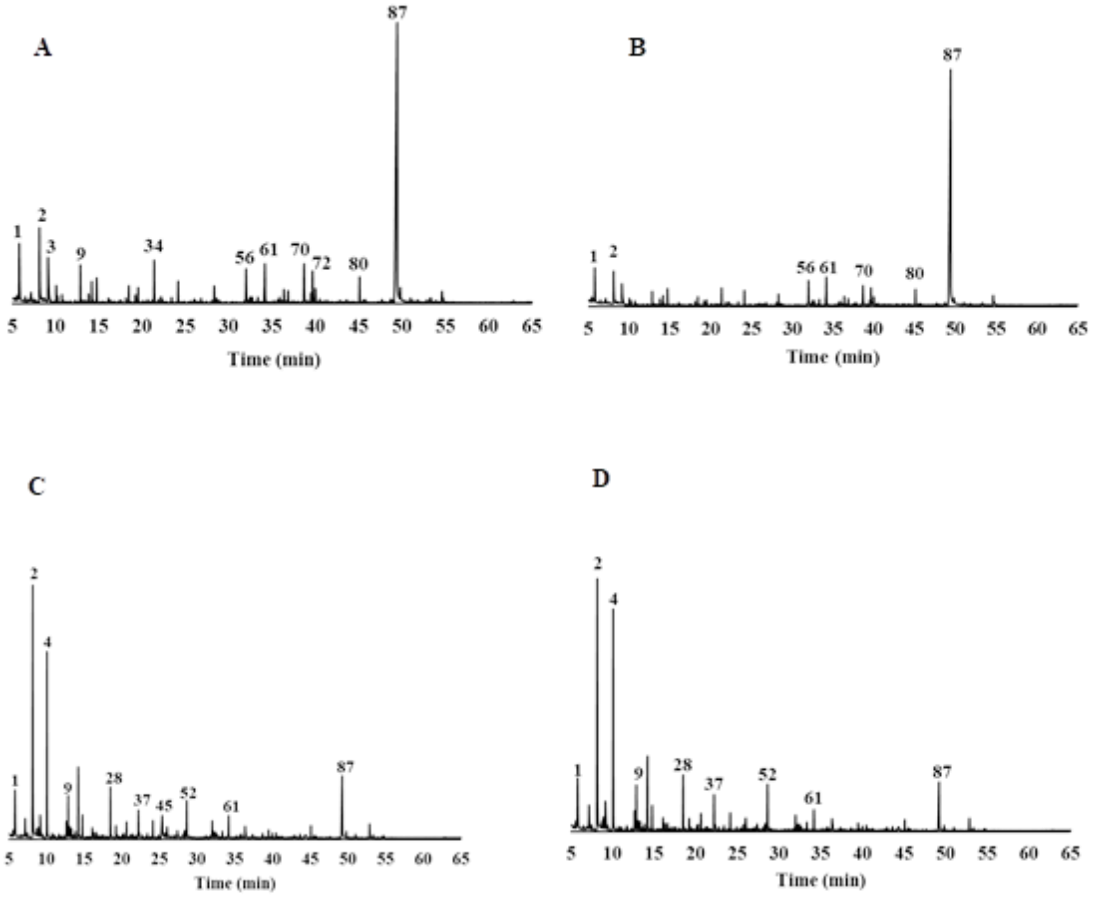


Fig. 4 Py-GC/MS chromatograms of the main delignified solids obtained by different delignification processes from CLRext a) CLRtreat for comparison; b) EO5 solid; c) AGO3 solid; d) ASP8 solid. The peak assignments of the derived pyrolysis products are given in Table 2.

Regarding the isolated lignins, the S/G ratio values were 1.9, 1.8, 1.0 and 1.3 for EO5, GO3, ASP7 and ASP8, respectively. Syringol derivatives were the dominant group in the pyrolyzed lignin samples of EO5, AGO3 and ASP7 (44.9%, 30.4% and 32.4% of the total lignin, respectively), with 4-vinylsyringol as the main constituent (10.5–13.2%), although guaiacol derivatives were obtained in similar proportion in ASP7 (32.8%,). Differences in the content of some compounds between ASP7 and ASP8 lignins were also observed, e.g. 4-vinylguaiacol (14.7% and 4%, respectively) and 4-vinylsyringol (11.7% and 5.7%, respectively). The main H-units were phenol (peak 42), p-cresol (peak 48) and 2,3-dihydrobenzofuran (peak 60), that represented from 6.3% of ASP7 to 9.7% of EO5). Other compounds with not determined lignin source (NDL) were also identified: toluene (peak 5), 1,3-dimethyl-benzene (peak 10), 1,4-dimethyl-benzene (peak 13) and styrene (peak 19), reaching a total of 6.4% in ASP7 lignin.

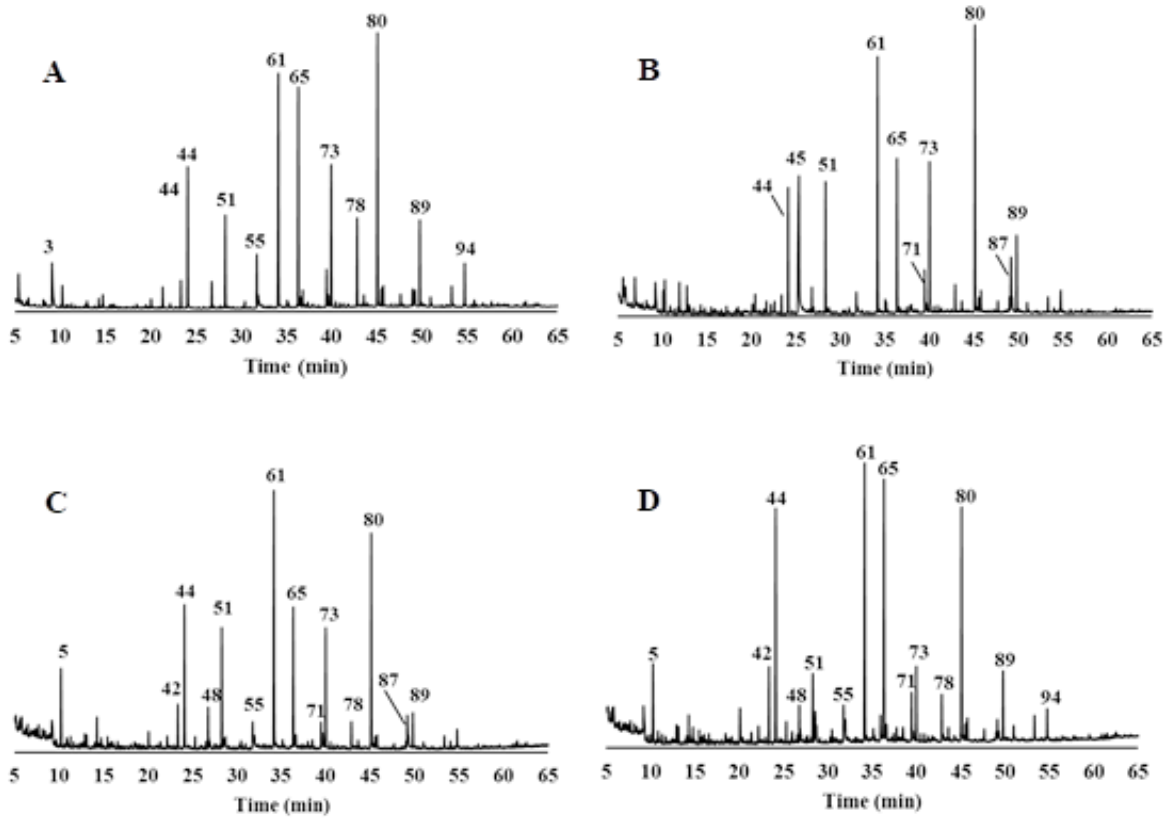


Fig. 5 Py-GC/MS chromatograms of the main lignins obtained from CLRext by different delignification processes. A) EO5 lignin; B) AGO3 lignin; C) ASP7 lignin; D) ASP8 lignin. The peak assignments of the derived pyrolysis products are given in Table 2.

The pyrograms of the delignified solids presented also some peaks originated from carbohydrates (cellulose and hemicellulose) (Fig. 4). The higher intensity of the cellulose products in these pyrograms is an effect of the selective fractionation of the delignification processes (Oudia et al., 2009). EO caused only a slight compositional change in the solid, while AGO and ASP produced solids with greater changes when compared to the CLRtreat e.g. hydroxyacetaldehyde (peak 2), 2-hydroxypropanone (peak 4) and 1,6-anhydro-β-D-glucopyranose (levoglucosan, peak 87). The pyrograms (Fig 4A and 4B) show that levoglucosan was the main carbohydrate-derived pyrolysis detected in CLRtreat and EO5 (42.7% and 51%, respectively), since it is the major product of cellulose pyrolysis (Li et al., 2001; Patwardhan et al., 2009; Lourenço et al., 2013b). This compound decreased sharply in ASP8 and AGO3 solids (dropped to ≤ 7%) (Fig 4C and 4D) and was quite reduced in all the lignin samples (≤ 3%) (Fig. 5 A-D). On the other hand, hydroxyacetaldehyde (peak 2) and 2-hydroxypropanone (peak 4) strongly increased in ASP8 and AGO3, which can be explained by the decline of levoglucosan associated to reactions involving opening and reforming of the pyranoid ring (Liao et al., 2004).

As expected, carbohydrates-derived pyrolysis products represented low percentages in the lignins isolated from the liquid stream (from 3.7% to 13.4%); levoglucosan was the main carbohydrate-derived product in GO3 (3.0%) and ASP7 (2.6%).

The results obtained showed that the type of the delignification process and the reaction time have an impact on the selectivity of the reactions involved and therefore on the type and composition of lignin-derived compounds present in the delignified solids and the solubilized lignins.

Table 2. Identification of the pyrolysis products (as % of total area) of the hydrothermally treated *Cistus ladanifer* residues (CLR treat), of delignified solid samples by the three processes - EO (sample EO5), AGO (sample AGO3) and ASP (sample S8) and of lignin precipitated from the delignification liquors of AGO (sample AGO3) and ASP (samples ASP7 and ASP8). The origin of the pyrolysis products is given as derived from carbohydrates (c), lignin hydrophenyl units (H), guaiacyl units (G), syringyl units (S) and undetermined lignin source (NDL) as well as from proteins (P) and other compounds (O).

			Hydrotermally Treated	Delignified solids			Lignin			
Peak nr	**Compound**	**Origin**	**CLR treat**	**EO5**	**AGO3**	**ASP8**	**EO5**	**AGO3**	**ASP7**	**ASP8**
1	2-oxo-propanal	c	3.7	3.4	3.7	3.8	0.0	1.3	n.d.	n.d.
2	hydroxyacetaldehyde	c	4.5	3.0	17.2	16.7	0.0	n.d.	n.d.	n.d.
3	acetic acid	c	3.8	2.5	2.0	2.4	3.7	1.9	n.d.	1.6
4	2-hydroxypropanone	c	0.8	0.5	9.3	10.8	0.0	0.9	n.d.	n.d.
5	toluene	NDL	0.3	0.4	0.2	n.d.	1.1	1.3	4.0	2.2
6	HOCH=CHOH	c	0.4	0.3	0.3	0.2	0.0.	n.d.	n.d.	n.d.
7	glycidol	-	n.d.	n.d.	0.1	n.d.	0.0	1.2	n.d.	n.d.
8	cyclopentanone	c	n.d.	n.d.	n.d.	n.d.	0.0	0.9	n.d.	n.d.
9	3-hydroxypropanal	c	1.4	0.8	2.1	2.4	0.0	n.d.	n.d.	n.d.
10	1,3-dimethyl-benzene	NDL	n.d.	n.d.	n.d.	n.d.	0.0	0.1	0.6	0.4
11	pyrrole	-	0.1	0.1	n.d.	n.d.	0.1	0.3	0.5	0.4
12	*trans* 2-methyl-but-2-enal	c	n.d.	n.d.	0.7	0.6	0.0	n.d.	n.d.	n.d.
13	1,4-dimethyl-benzene	NDL	n.d.	n.d.	n.d.	n.d.	0.0	n.d.	0.5	0.4
14	3*H*-furan-2-one	c	0.1	0.1	0.6	0.5	0.0	n.d.	n.d.	n.d.
15	furan-2-one isomer	c	0.3	0.3	0.2	0.2	0.0	n.d.	n.d.	n.d.
16	3-furaldehyde	c	0.3	0.3	0.2	0.2	0.0	0.0	0.0	0.0
17	CH_3-CO-CHOH-CHO	c	1.0	0.4	2.4	2.6	0.0	0.0	0.0	0.0
18	CHO-CH_2-CH_2-CHO	c	n.d.	0.4	2.4	2.6	0.0	0.0	0.0	0.0
19	styrene	NDL	0.1	0.2	n.d.	n.d.	0.4	0.3	1.3	0.6
21	furfural	c	1.2	0.7	0.8	0.8	0.2	0.0	0.0	0.0
22	2-cyclopenten-1-one	c	0.1	0.7	0.8	0.8	0.2	0.0	0.4	0.1
23	5-methyl-3*H*-furan-2-one	c	n.d.	n.d.	0.4	n.d.	0.0	n.d.	n.d.	n.d.
24	furfuryl alcohol	c	0.3	0.3	0.4	0.8	0.0	n.d.	n.d.	n.d.
25	4-cyclopentene-1,3-dione	c	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.1
26	similar to 4-cyclopentene-1,3-dione	c	0.1	0.1	0.2	0.2	0.0	0.0	0.0	0.2
27	dihydro-4-hydroxy-3*H*-furan-2-one,	c	0.2	0.3	n.d.	n.d.	0.0	n.d.	n.d.	n.d.
28	2-hydroxy-2-cyclopenten-1-one	c	0.8	0.6	2.7	2.9	0.2	0.3	0.0	0.3
29	dihydro-methyl furanone isomer	c	0.5	0.4	0.8	0.7	0.0	0.0	0.0	0.0

30	5-methyl-2-furaldehyde	c	0.2	0.2	0.2	0.1	0.0	0.0	0.0	0.0
31	Not identified sugar	c	0.7	0.4	0.1	0.1	0.2	0.0	0.0	0.0
32	Not identified sugar	c	n.d.	n.d.	n.d.	n.d.	0.0	0.8	n.d.	0.3
33	5*H*-furan-2-one	c	0.2	0.2	1.0	1.0	0.0	0.0	0.0	0.0
34	4-hydroxy-5,6-dihydro-2*H*-pyran-2-one	c	2.1	1.3	0.2	0.2	1.1	0.3	0.0	0.3
35	2-ethenyl-1,3-dioxolane-4-methanol	c	n.d.	n.d.	n.d.	n.d.	0.0	0.5	n.d.	n.d.
36	2*H*-pyran-2-one	c	0.3	0.3	0.1	0.1	0.0	0.0	0.0	0.0
37	2-hydroxy-3-methyl-2-cyclopenten-1-one	c	0.2	0.2	0.9	2.1	0.0	n.d.	n.d.	n.d.
38	methyl-dihydro-2*H*-pyran-2-one	c	0.2	0.2	0.9	n.d	0.0	n.d.	n.d.	n.d.
39	2-hydroxy-1-methyl-1-cyclopentene-3-one isomer	c	0.3	0.4	0.3	0.3	0.0	n.d.	n.d.	n.d.
40	Not identified sugar	c	n.d.	n.d.	0.3	0.3.	0.0	n.d.	n.d.	n.d.
41	similar to 2-ethenyl-1,3-dioxolane-4-methanol	c	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0
42	phenol	H	0.3	0.3	0.1	0.3	1.3	0.7	2.2	1.8
43	2-(propan-2-one)-tetrahydrofuran	c	0.5	0.5	0.5	0.1	0.0	2.2	n.d.	n.d.
44	guaiacol	G	0.5	0.5	0.5	1.0	6.2	2.2	7.1	5.7
45	glycerin	O	n.d.	n.d.	1.1	n.d.	0.0	12.7	n.d.	n.d.
46	*o*-cresol	H	n.d.	n.d.	1.1	n.d.	0.0	n.d.	0.7	0.6
47	3-ethyl-2-hydroxy-2-cyclopenten-1-one	c	n.d.	n.d.	1.1	0.3	0.0	n.d.	n.d.	n.d.
48	*p*-cresol	H	0.3	0.2	0.1	0.04	1.2	0.5	2.1	0.9
49	*m*-cresol	H	n.d.	n.d.	n.d.	0.1	0.0	0.5	0.4	0.4
50	2-methoxy-6-methylphenol	H	n.d.	n.d.	n.d.	0.1	0.0	0.3	n.d.	0.3
51	4-methylguaiacol	G	0.9	0.9	0.4	0.4	4.2	4.5	6.4	1.8
52	Not identified sugar	c	0.3	0.2	2.5	3.1	0.0	0.3	0.7	1.0
53	2,3-dimethyl-phenol	H	0.1	n.d.	0.1	0.1	0.0	0.2	0.9	0.6
54	ethylphenol	H	n.d.	n.d.	n.d.	n.d.	0.4	n.d.	n.d.	n.d.
55	4-ethylguaiacol	G	n.d.	0.1	0.2	0.2	2.2	0.7	1.4	1.1
56	Not identified sugar	c	2.1	2.2	1.2	1.0	0.0	n.d.	n.d.	0.9
57	Similar to dihydro-6-methyl-2*H*-pyran-3(4*H*)-one	c	0.2	0.1	0.5	0.5	0.0	0.0	0.0	0.0
58	3,4-anhydro-D-galactosan	c	0.4	0.4	0.1	n.d.	0.0	n.d.	n.d.	n.d.
59	1,4:3,6-dianhydro-α-D-glucopyranose	c	0.3	0.5	0.5	0.5	0.0	n.d.	n.d.	n.d.
60	2,3-dihydrobenzofuran	H	n.d.	0.6	0.1	n.d.	6.8	4.9	n.d.	4.0
61	4-vinylguaiacol	G	1.4	2.4	1.3	1.3	6.8	4.9	14.7	4.0
62	eugenol	G	0.1	0.1	0.1	0.2	0.3	0.4	0.4	0.4

63	4-propylguaiacol	G	n.d.	n.d.	n.d.	n.d.	0.2	n.d.	n.d.	n.d.
64	5-hydroxymethylfurfural	c	0.3	0.5	0.2	0.2	0.0	0.0	0.0	0.0
65	syringol	S	0.8	0.7	0.7	0.5	10.8	5.5	7.5	6.9
66	indole	P	0.1	n.d.	n.d.	n.d.	0.6	0.2	0.4	0.6
67	Not identified sugar	c	0.6	0.5	n.d.	n.d	1.0	n.d.	n.d.	n.d.
68	dihydro-4-hydroxy-3*H*-furan-2-one	C	n.d.	n.d.	0.2	0.2	0.0	n.d.	n.d.	n.d.
69	*cis* isoeugenol	G	n.d.	n.d.	0.2	0.1	0.2	0.3	n.d.	0.2
70	2-hydroxymethyl-5-hydroxy-2,3-dihydro-4*H*-pyran-4-one	c	2.8	2.1	0.4	0.1	0.0	0.0	0.0	0.0
71	*trans* isoeugenol	G	0.5	0.4	0.5	0.5	1.8	1.7	1.4	1.3
72	similar to 1,5-Anhydro-arabinofuranose	c	2.3	1.8	0.1	0.1	1.1	0.6	0.0	0.0
73	4-methylsyringol	S	1.0	0.8	0.4	0.2	6.8	5.7	6.2	2.0
74	vanillin	G	0.2	0.2	0.4	0.4	0.3	0.3	n.d.	0.3
75	1-(4-hydroxy-3-methoxyphenyl)-propyne	G	0.1	n.d.	0.1	n.d.	0.2	0.3	n.d.	0.2
76	1-(4-hydroxy-3-methoxyphenyl)-propyne	G	0.1	0.1	n.d.	n.d.	0.2	0.2	n.d.	n.d.
77	homovanillin	G	0.1	0.1	0.2	0.1	0.0	0.0	0.0	0.0
78	4-ethylsyringol	S	0.1	0.1	0.1	0.1	4.1	1.0	1.4	1.3
79	acetoguaiacone	G	0.1	0.1	0.3	0.3	0.6	0.4	0.6	0.4
80	4-vinylsyringol	S	1.2	1.2	0.8	0.6	13.2	10.5	11.7	5.7
81	guaiacylacetone	G	0.1	0.1	0.2	0.2	0.9	0.6	0.8	0.5
82	4-allylsyringol	S	0.1	0.1	0.1	0.1	1.0	0.4	0.4	0.3
83	4-propylsyringol	S	0.1	0.1	0.1	0.1	0.0	0.4	0.4	0.3
84	*trans* coniferyl alcohol	G	n.d.	n.d.	0.1	n.d.	0.0	n.d.	n.d.	n.d.
85	*cis* 4-propenylsyringol	S	0.2	n.d.	0.1	n.d.	0.6	0.4	n.d.	0.3
86	4-propinylsyringol	S	0.1	n.d.	0.1	n.d.	0.8	0.7	0.4	0.3
87	1,6-anhydro-β-D-glucopyranose	c	42.7	51.0	7.0	4.8	0.9	3.0	2.6	0.8
88	4-propinylsyringol	S	n.d.	n.d.	n.d.	n.d.	0.9	0.9	0.9	0.3
89	*trans* 4-propenylsyringol	S	0.8	0.6	0.5	0.3	3.9	2.9	1.9	1.8
90	syringaldehyde	S	0.2	0.2	0.2	0.2	0.4	0.4	n.d.	0.4
91	Not identified compound	-	n.d.	n.d.	1.1	1.0	0.0	n.d.	n.d.	n.d.
92	acetosyringone	S	0.3	0.2	0.2	0.2	1.0	0.7	0.7	0.6
93	*trans* coniferaldehyde	G	n.d.	0.6	0.2	n.d	0.0	n.d.	n.d.	n.d.
94	syringylacetone	S	0.2	0.2	0.2	0.1	1.9	0.9	0.9	1.0
95	propiosyringone	n.d.	n.d.	n.d.	n.d.	n.d.	0.3	n.d.	n.d.	n.d.
96	*trans* sinapaldehyde	S	0.2	n.d.	0.1	n.d.	0.0	n.d.	n.d.	n.d.
		S	5.1	4.2	3.6	2.4	44.9	30.4	32.4	21.2

	G	4.2	5.6	4.7	4.7	24.0	16.5	32.8	15.9
	H	0.6	1.1	1.5	0.6	9.7	7.1	6.3	8.6
	NDL	0.5	0.6.	0.2	n.d.	1.5	1.7	6.4	3.6
	S/G	1.2	0.8	0.8	0.5	1.9	1.8	1.0	1.3
	S:G:H	1 : 0.8 : 0.1	1 : 1.3 : 0.3	1 : 1.3 : 0.4	1 : 2 : 0.3	1:0.5: 0.2	1 : 0.5 : 0.2	1: 1 : 0.2	1 : 0.8 : 0.4
Total lignin (% identified area)		10.4	11.5	10.0	7.7	68.7	55.7	77.9	49.3
Total carbohydrates (% identified area)		76.3	78.2	65.6	64.4	7.6	13.4	3.7	5.6

4. Conclusions

Delignification of *Cistus ladanifer* residues after hydrothermal treatment by organosolv processes was more effective with the alkali-catalyzed glycerol organosolv process than with the ethanol organosolv which preserved the residual cellulose (> 88%) but exhibited lower enzymatic saccharification yields due to the low delignification achieved. The aqueous sodium hydroxide process was the most efficient for the selective separation of lignin and cellulose as well as for the enzymatic hydrolysis of the delignified biomass.

The delignification reactions were selective regarding the different lignin units with S-units being preferentially removed, therefore leaving residual lignins in the solids that were enriched in G- and H-units, the effect being highest for the soda process. Low molecular weight phenolic compounds were also present in all the delignification liquors and may have a potential use as bioactive agent.

Cistus ladanifer, besides being a recognized source of essential oil, can be further processed and integrated as a biorefinery feedstock allowing production of lignin-derivatives and glucan-rich solids to be used for the production of other added-value products.

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CAPÍTULO VII

D-lactic acid production from *Cistus ladanifer* residues: co-fermentation of pentoses and hexoses by *Escherichia coli* JU15

A informação deste capítulo tem como base a seguinte publicação:

Júnia Alves-Ferreira, Florbela Carvalheiro, Luís C. Duarte, Ana R. P. Ferreira, Alfredo Martinez, Helena Pereira, Maria C. Fernandes (2019). D-lactic acid production from *Cistus ladanifer* residues: co-fermentation of pentoses and hexoses by *Escherichia coli* JU15. (em submissão)

Abstract

In this study, glucan-rich solids and xylose-rich hydrolysates from *Cistus ladanifer* distillery residues (CLR) were used for lactic acid (LA) production by the recombinant strain *Escherichia coli JU15*. Firstly, hydrolysates obtained by autohydrolysis process were submitted to sulfuric acid–catalyzed post-hydrolysis under various acid concentrations and reaction times. The optimal condition for post-hydrolysis was found with 3% H_2SO_4 for 15 min at 121 ºC. Subsequent detoxification procedures were carried out over post-hydrolysed liquors, where 10% (w/v) powdered activated charcoal it enabled a reduction of acetic acid (37%), formic acid (27%), whereas HMF, furfural and phenolic compounds were completely eliminated. The diverse fermentation modes using detoxified and non-detoxified spent liquors, as well as using glucan-rich solids previously delignified with NaOH alone (SHF or SSF) or together with pentoses liquors (SSCF) (5% loading) were performed and for all the tested conditions the product yield ($Y_{LA/sugars}$) always surpassed 92 g/g.

Keywords: Hemicellulosic hydrolysates, Detoxification, Glucan-rich solid, Saccharification, D-lactic acid

1. Introduction

Cistus ladanifer is a widespread shrub in the Iberia southern territory and an important source of high-value essential oils for the cosmetics and perfume industry. Most of the products from *C. ladanifer* (labdanum gum, essential oils and concrete) are produced in Spain (c.a. 80%), mainly in Andalusia (Biolandes, 2018). The yields obtained from the different extraction processes to obtain the CL products are low (c.a. 6% for gum and 1% for essential oil) and the residues (CLR) generated are only used as low value-added solid (i.e. burning, cogeneration energy and composting). However, our recent works have proposed an integrated process for the fractionation of CLR, which include the sequential separation of extractives, hemicellulosic sugars (as oligosaccharides) and lignin to yield value-added products shown that distillery residues have the potential to be valorized in a biorefinery (Alves-Ferreira, 2017; 2019).

A biorefinery can be defined as an "industrial installation that integrates processes and equipment for the conversion of biomass to produce fuels, energy and a series of value-added chemical products with the minimum of waste and emissions" (Demirbas, 2009). Among the products that can be obtained in a biorefinery, organic acids are very relevant. Latic acid (LA) is an example of organic acid widely used in food and non-food industries (cosmetic, pharmaceutical, and chemical), but it is expected that its use for the production of biodegradable and biocompatible polylactic acid (PLA) polymers will become its strongest application (Martinez et al., 2017; Abdel-Rahman et al., 2013; Wang et al., 2013a).

LA can be produced by chemical or fermentative routes. The fermentative route has advantages such as the use of renewable materials and the obtaining of the optically pure D- or L-lactic acid when the appropriate microorganism is selected (Abdel-Rahman et al., 2013). Several bacterial strains, in particular from *Lactobacillus genus*, have been described to have the ability to produce mixtures of L-lactic acid and D-lactic acid (D-LA), though the exclusive production of D-LA is only reported in a limited number of studies (Singhvi et al., 2017; Hama et al., 2015; Zhang and Vadlani, 2013). Furthermore, the majority of *Lactobacillus* strains are unable to utilize pentose sugars as the carbon and energy sources, which limits the possibility to fully use the polysaccharides in lignocellulosic biomass. Thus, the genetic engineering tools have been applied in the search for the improvement of lactic acid yield and optical purity by microbial producers (Abdel-Rahman et al., 2013). In this context, *Escherichia coli strain* JU15, developed by metabolic engineering and adaptive evolution, have been reported as a D-lactic acid producer from hexose and pentose sugars (Utrilla et al., 2009 and 2012).

In general, after the pretreatment of the biomass (hydrolysis of hemicellulose and/or delignification), the production of LA can be performed from hemicellulosic hydrolysates or glucan-rich solids remaining after these treatments. This way, the main fermentative processes used for obtaining lactic acid are Separate Hydrolysis and Fermentation (SHF). Nevertheless, the co-fermentation can also be considered when are used strains with the ability to utilize both hexoses and pentoses. These processes are called separate hydrolysis and co-fermentation (SHCF) or Simultaneous Saccharification and Co-Fermentation (SSCF). The latter process is the most desirable because it allows the hydrolysis and fermentation of sugars in the same process. However, this strategy requires robust microorganisms, preferably thermotolerant strains as the optimum temperature for the enzymatic saccharification (50 °C) in general is higher than the optimum temperature for growth of microorganisms, which makes the SS(C)F process more difficult to control. Regarding to hydrolysates, the presence of inhibitory compounds, such as furan derivatives, aliphatic acids and phenolic compounds, can be a challenge for the fermentative microorganisms, emphasizing that recombinant strains might be more sensitive to toxic compounds present in the hydrolysates than wild type strains (Taherzadeh et al. 1997; Zaldivar and Ingram, 1999; Zaldivar et al., 1999; Palmqvist et al, 1999). Nevertheless, there are several possible approaches to reduce the effects of inhibitors, that include the implementation of hydrolysis conditions in which their formation is minimized to adapt microorganisms to the hydrolysates or make their removal before fermentation (detoxification), if necessary. The possible detoxification methods include treatments with activated charcoal or ion exchange resins, solvent extraction, "overliming", detoxification with membranes (nanofiltration) (Martinez et el., 2001; Carvalheiro et al., 2005; Santos et al., 2011, Mitchell et al., 2013).

Thus, this work aimed to the development of a methodological approach for the production of D-LA by the recombinant strain *E. coli* JU15 from both the glucan rich solids and pentose hydrolysates resulting from pretreatment processes of *C. ladanifer* distillery residues (autohydrolysis and delignification) under conditions previously studied (Alves-Ferreira et al., 2019 and submitted). Liquors were firstly subjected to post-hydrolysis with diluted sulfuric acid under various concentrations and reaction times. Sequentially, the liquors subjected to optimal conditions of post-hydrolysis were detoxified with calcium oxide (CaO; for pH adjustment) and two types of activated charcoals to evaluate the most efficient procedure in the removal of inhibitors. Lastly, diverse fermentation modes, namely SHF, SSF and SHCF as well as the fermentations using detoxified and non-detoxified (pH adjusted) hydrolysates were evaluated. The development of an integrated upgrading strategy for *C. ladanifer* distillery residues (CLR), a by-product from the

essential oil industry, can be an important option for the use of this biomass in a biorefinery framework.

2. Material and methods

2.1. Hemicellulosic hydrolysates and pretreated solids

The hemicellulosic hydrolysate used in this work was produced from CLR previously distilled to obtain essential oils and extracted successively in Soxhlet with ethanol and water (CLRext). For the selective hydrolysis of hemicellulose, the raw material was treated by autohydrolysis process in a 600 mL stainless steel reactor (Parr Instruments), under non-isothermal conditions at 205 ºC, as detailed in Alves-Ferreira et al. (2019). The hemicellulosic liquor obtained from the hydrothermal process had a pH of 3.6 and contained in g/L: oligosaccharides, 23; monosaccharides, 6.8; aliphatic acids, 2.7; furan derivatives 0.5 and total phenolic compounds 3.4.

The remaining solids obtained under a previously optimized hydrothermal condition (CLR treat) were subjected to delignification treatment with 4% NaOH, in a solid-to liquid ratio of 1:10 (w:v), at 130 ºC for 1 h as determined by a procedure described previously (Alves-Ferreira et al. (submitted)). The chemical composition of glucan rich solid obtained after delignification treatment was 73.7% glucan, 5.97 xylan, 16.8% lignin and 3.2% ash.

2.2. Post-hydrolysis

In order to evaluate the post-hydrolysis conditions that lead to the highest production of monomeric sugars, the oligosaccharides-containing hydrolysates were subjected to an acid post-hydrolysis. Sulfuric acid was added to hemicellulosic liquor in order to obtain final concentrations of 1, 2, 3 and 4% (w/w) in the reaction medium. The hydrolysates were performed in an autoclave at 121 ºC for 15 or 60 min and were done at least in duplicate.

2.3. Detoxification procedures

The effect of detoxification was evaluated in the liquors obtained under optimized conditions of the post-hydrolysis.

2.3.1. Liming process (pH adjustment)

The pH of the hydrolysate was corrected to 7 (fermentation pH) by the addition of CaO. After 1 h of stirring, precipitate solids were removed by centrifugation (5000 rpm, 15 min).

2.3.2. Activated charcoal treatment

For these treatments, two different types of charcoal were used: granulated (ca. 2.5mm, Merck, Germany) and activated powder (Panreac, Germany). Firstly, granular activated charcoal was washed with distilled water and dried at room temperature. Then it was equilibrated with HCl 0.4 M for 1 h, washed with distilled water and again dried at room temperature for c.a. 48 h. Both granulated and activated powder charcoal were mixed with the hydrolysates obtained after post-hydrolysis (1:10 (w/v)) and agitated for 1 h at room temperature. The detoxified hydrolysates were vacuum filtrated using Whatman no. 1 filter paper and the pH was adjusted to 7 with CaO or H_2SO_4.

2.4. Microorganisms and media

The fermentation studies were carried out using the genetically modified *E. coli* JU15 strain (Utrilla et al., 2012). AM1 medium (Martinez et al., 2007) was used for cell growth and inoculum preparation (Utrilla et al., 2012 and 2016). This medium contained: 2.63 g/L $(NH_4)2HPO_4$, 0.87 g/L $NH_4H_2PO_4$, 1.5 mL/L $MgSO_4.7H_2O$ (1 M), 1.5 mL/L trace elements, 1.0 mL/L KCl (2 M), 1.0 mL/L betaine-HCl (as osmoprotectant) (1 M). The medium was supplemented with 4 g/L of sodium acetate and 1.0 mL/L Kanamycin (30 mg/mL). The trace elements solution contains per liter: 1.6 g $FeCl_3$, 0.2 g $CoCl_2.6H_2O$, 0.1 g $CuCl_2$, 0.2 g $ZnCl_2.4H_2O$, 0.2 g Na_2MoO_4, 0.05 g H_3BO_3 and 0.33 g $MnCl_2.4H_2O$. The medium was sterilized using 0.22 mm membrane filters, except non-detoxified hydrolysate that was sterilized by autoclaving (121 ºC, 15 min), since these ones caused clogging of the filters making sterilization difficult.

The JU15 strain used in this study was stocked at -80 °C in cryovials containing 1 mL of glycerol (80%) and 1 mL of cells grown in AM1 medium containing xylose as carbon source. Pre-inoculum was prepared by re-suspending the cells in test tubes containing 3 mL of sterile Luria Broth (LB) (20 g/L) and 3 µL of kanamycin and incubated in an orbital shaker (200 rpm) at 37 ºC for 1 h. For inoculum preparation, the pre-inoculum content was transferred to a non-aerated mini-fermenter with 200 mL of sterile AM1 mineral medium supplemented with 20 g/L of glucose or xylose and performed at 37 ºC and 100 rpm. The initial pH was adjusted to 7.0 and controlled by the automatic addition of KOH (2 N). Fermentations with mineral AM1 medium were performed in order to better understand the performance of JU15 strain before the fermentations with hydrolysate-based media.

2.5. Fermentation

For the production of D-lactic acid, both pentose hydrolysates used alone or in combination with the cellulose-rich solids were used in the fermentation process, establishing the co-fermentation. Thus, hemicellulosic hydrolysates were used as fermentation medium, either detoxified or non-detoxified. Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF), and Simultaneous Saccharification and Co-Fermentation (SSCF) were tested for the delignified cellulose-rich solids in combination with the detoxified hemicellulosic hydrolysates

The pre-inoculum and inoculum containing xylose (20 g/L) as carbon source were prepared as described in section 2.4 and incubated for 24 h for seed culturing. The cells were harvested by centrifugation (5000 rpm, 10 min at room temperature), and used to inoculate the bioreactor containing 200 mL medium culture at an initial optical density (OD) of 0.2 at 600 nm (c.a. 0.076 g_{DCW}/L) and incubated under the same conditions as the inoculum. Stirring was fixed at 100 rpm for hydrolysate-based media and 200 rpm for experiments containing glucan-rich solids. To avoid microbial contamination, 30 mg/L of kanamycin were added to all the assays. The pH (7) was kept constant by automatic addition of KOH (2N). At pre-established times, samples were withdrawn for HPLC analysis. All experiments were performed, at least, in duplicate. The completion of the experiments occurred after pH stop to decrease.

The different types of fermentation were carried out as follows: CLRext hemicellulosic liquors were hydrolysed using the optimised condition in this work (3% sulfuric acid (w/w) at 121 ºC for 15 min). In a subsequent step, hydrolysates were subjected to detoxification processes with activated charcoal for comparison of

fermentative performance in relation to non-detoxified hydrolysates. The detoxification was performed according to the procedure mentioned in section 2.2 using activated charcoal powder.

The pH of both detoxified and non-detoxified liquors was adjusted to 7 with CaO. The detoxified hydrolysates were sterilized using membrane filters (0.22 mm) and the non-detoxified ones by autoclave at 121 ºC for 15 min. For non-detoxified hydrolysates, it was not possible to carry out sterilization by filtration due to the excess clogging of filters. After sterilization, the liquors were supplemented with the compounds aforementioned for AM1 medium. However, sodium acetate was not added due to the natural presence of sodium acetate (acetic acid) in the hydrolysates, whereas citric acid (0.1 g/L) was added to minimize salts precipitation. Before inoculation, when necessary, the pH was adjusted to 7 with 6 N KOH. The hydrolysates were inoculated, as described above, with the strain JU15 and the fermentations were monitored for up to 108 h.

Glucan-rich solids were used in the fermentative experiments applying SHF and SSF processes. In the SHF process, enzymatic hydrolysis and fermentation were carried in two different steps, where each one was performed at the optimal conditions of pH and temperature. Saccharification was performed using a solid loading of 5% in 200 mL mini-fermenter at 50 ºC for 72 h and 200 rpm. The solid load was defined according to the stirring capacity of the system and size of the mini fermenter. 20 FPU/g biomass of a commercial enzymatic cocktail (Cellic C-Tec2, Novozymes) and 50 mM sodium citrate buffer (pH 5) were used. The enzymatic activity (257 FPU/mL) was determined using filter paper (Whatman No. 1) according to Ghose (1987). After 72 h, in the same vessels used for saccharifications, AM1 nutrients, as well as 4 g/L of sodium acetate as supplement were added. The initial pH was adjusted to 7 with KOH (6N) and cultures were inoculated with strain JU15 according to the already described conditions. In the SSF process an enzymatic pre-hydrolysis (12 h) was performed, followed by inoculation with JU15 in the same conditions described for SHF. For SSCF, the glucan-rich solids and detoxified hydrolysates are combined and used for the production of D-lactic acid simultaneously with the enzymatic hydrolysis. Similarly, to SSF, an enzymatic prehydrolysis was also carried out before inoculation. For this process, no sodium acetate was added in the culture medium, due to the presence of acetic acid from hydrolysates. The fermentation was performed as described for other assays.

2.6. Analytical methods

Glucan, xylan, acetyl groups, Klason lignin, and ash content of the remaining solids from pretreatments and fermentations were determined on the basis of the NREL procedures (Sluiter et al., 2008). The analysis of monosaccharides in the hydrolysate were carried out by HPLC, using a Refractive Index detector (Waters 2410), an ultraviolet (UV) detector (Waters 486) and a BioRad Aminex HPX-87H column (Bio-Rad, USA), with injection of a 20 µL sample, sulfuric acid (5 mM) as eluent, at 0.4 mL/min flow rate, oven temperature of 50 ºC. The analysis of monosaccharides, aliphatic acids and furan derivatives and D-lactic content in the hemicellulosic hydrolysates and fermentation media were also analyzed by HPLC as detailed elsewhere (Fernandes et al., 2018). Samples were filtered through 0.22 µm membranes before HPLC analysis. Total amount of phenolics were determined by the Folin–Ciocalteu colorimetric method using a microplate spectrophotometer (MultiskanTM GO, Thermo Scientific, USA) adapted by Roseiro et al. (2014) and detailed in (Alves-Ferreira et al., 2019). Cell growth was evaluated by measuring the optical density at 600 nm using a spectrophotometer (Helios Alpha, Thermo Scientific, USA).

2.7. Calculations

The calculations for the specific growth rate (µ, h^{-1}), volumetric productivity of D-lactic acid (Q_{DLA}: g/Lh) and D-lactic acid yield ($g_{D\text{-}LA}/g_{Sugars}$) were accomplished as described in Carvalheiro et al. (2011): the specific growth rate of lactogenic *E. coli* JU15 in minimal medium with glucose or xylose (µ, h^{-1}), was calculated by linear regression of the curve of optical density versus time for the exponential growth phase. The volumetric productivity of D-lactic acid (Q_{DLA}) was based on the maximum lactic acid produced at designated times. The lactic acid yield ($Y_{D\text{-}LA/Sugars}$) was calculated as the ratio between the maximum D-lactic acid concentration and sugars consumption during that fermentation period.

3. Results and discussion

3.1. Post-hydrolysis of hemicellulosic hydrolysates

The hemicellulosic oligosaccharides obtained in liquid fraction of the hydrothermal treatment were subjected to dilute acid post-hydrolysis using different concentrations of the catalyst (sulfuric acid) and reaction times. The results obtained are shown in Table 1. These results allowed an evaluation of the best condition to obtain a high monomers concentration in comparison to the standard treatment of acid hydrolysis (4%, 60 min) indicating an economy with the catalyst and reaction time.

The concentration of monosaccharides varied from 18.6 g/L (run D) to 29.6 g/L (run G). Run E correspond to standard condition. For run D, no gluco-oligosaccharides (GOS) hydrolysis was observed. In run C there was a slight decrease in the hydrolysis of GOS in relation to other runs (6.4 g/L), which in turn showed similar levels of glucose (7.6–7.9 g/L). This behaviour indicates a higher difficult to hydrolyse of GOS and have been discussed elsewhere (Duarte et al., 2004).

Run D also presented a lower hydrolysis of xylo-oligosaccharides (XOS) to xylose compared to the other runs. Nevertheless, it is possible to observe also a negative impact in xylose recovery in the standard test (run E) in relation to the most conditions it may be due to its degradation, as indicated by a slight increment in the furfural concentration. In general, arabinose content maintained a similar concentration to the initial liquor under all conditions tested (about 3.8%). This indicates that arabinan was almost completely converted into monomers during the autohydrolysis. Furfural concentration, increased with time and acid concentration; HMF remained constant in all conditions; formic acid content varied between 1.1. and 1.5 g/L without presenting a linear trend; acetic acid exhibited similar concentrations (from 4.9 to 5.2%).

This way, taking into account the similar chemical composition presented by the tests B and G, experiment B (3% sulfuric acid and 15 min hydrolysis time) should be considered the most adequate condition for acid post-hydrolysis, once the lower time requirements can considerably reduce energy costs. In fact, increasing catalyst also cause an impact on process economics, but the small difference in catalyst concentration between these treatments does not compensate the longer hydrolysis time required by test G.

Table 1. Operational conditions and composition of liquors (monosaccharides. aliphatic acids and furan derivatives) obtained after sulfuric acid–catalyzed post-hydrolysis

	Operational conditions		**Composition (g/L)**							
Exp.	Time (min)	H_2SO_4 (% w/w)	Glucose	Xylose	Arabinose	Total Monosaccharides	Acetic acid	HMF	Furfural	Formic
Hydrolysate	-	-	1.0 (6.7)[a]	2.1 (15.8)[a]	3.7 (0.40)[a]	6.8	1.6	0.1	0.4	1.1
A		4	7.6±0.0	17.4±0.2	3.8±0.0	28.8	5.0±0.1	0.1±0.0	0.7±0.0	1.2±0.1
B	15	3	7.6±0.1	17.8±0.2	3.8±0.0	29.2	5.1±0.1	0.1±0.0	0.7±0.0	1.1±0.1
C		2	6.4±0.3	16.4±0.6	3.8±0.1	26.6	4.9±0.2	0.1±0.0	0.6±0.0	1.3±0.1
D		1	1.0±0.0	13.8±0.5	3.8±0.0	18.6	4.9±0.2	0.1±0.0	0.5±0.0	1.4±0.0
E		4	7.6±0.0	17.2±0.2	3.7±0.0	28.5	5.0±0.0	0.1±0.0	1.0±0.0	1.5±0.1
F	60	3	7.8±0.0	17.5±0.0	3.7±0.0	29.0	5.1±0.0	0.1±0.0	0.9±0.0	1.5±0.0
G		2	7.9±0.0	17.9±0.0	3.8±0.0	29.6	5.2±0.2	0.1±0.0	0.8±0.0	1.2±0.0
H		1	7.8±0.1	17.7±0.1	3.8±0.1	29.3	5.2±0.3	0.1±0.8	0.8±0.0	1.3±0.0

[a] Value in parenthesis indicates the oligosaccharides concentration in g/L

3.2. Effect of detoxification on the chemical composition of the hydrolysates

According to the previous post-hydrolysis results, under the best conditions the hydrolysate contained about 30 g/L of total monosaccharides, being c.a. 74% constituted by pentose sugars. Acetic acid and formic acid represent 5.1 g/L and 1.1 g/L, respectively. Furfural is the major furan derivative due to pentose degradation, whereas total phenolics represent 3.3 g/L. The results obtained for sugar and inhibitor concentrations after the liming process (pH adjustment) and two different types of activated charcoal are summarized in Table 2.

The liming using CaO had a negligible effect on the loss of sugars being that furan derivatives and aliphatic acids remained unchanged. Conversely, there was a decrease of 48.5% in the content of phenolic compounds. A slight effect on the reduction of inhibitors and on recovery sugars of brewery's spent grain hydrolysate resulting of pH adjustment to 5.5 was previously reported (Carvalheiro et al., 2005). On the other hand, a study lead by Mateo et al. (2013) showed an important effect of liming detoxification using CaO (5.5 pH) on olive tree pruning hydrolysates residue. A simultaneous reduction of sugars amount (15%), furans (56%), phenolic compounds (62%) and acetic acid (32%) was described.

Table 2. Effect of detoxification treatments on the composition of the CLRext post-hydrolysis liquors

Exp.	Hydrolysate treatment	Xylose	Glucose	Arabinose	Acetic acid	Formic acid	Furfural	HMF	Phenolics
1	None	17.8	7.6	3.8	5.1	1.1	0.7	0.1	3.3
2	Liming (pH 7)	17.7	7.5	3.6	5.0	1.1	0.7	0.1	1.7
3	Actived charcoal granulated [a]	16.4	6.8	3.5	4.3	0.9	0.2	0.1	0.8
4	Actived charcoal powder	14.8	6.3	3.2	3.2	0.8	0.0	0.0	0.0

[a] Equilibrated with HCl

Activated charcoal treatment using granulated charcoal equilibrated with HCl led to a 8% loss of total monosaccharides, while a reduction of 16%, 18%, 71% and 75% in the acetic acid, formic acid furfural and phenolic compounds content, respectively was obtained. These results agreement with those of Carvalheiro et al (2005) using this same activated charcoal, where a loss of 13% for total sugars, 11–17% in the aliphatic acids removal and an important reduction of furan derivatives (92–68%) was attained. The treatment with activated charcoal powder was the most efficient in the removal of

inhibitors, but also the one that caused the highest loss of sugars (c.a. 16%). Works using a lower charge of activated charcoal (2.5%) had minimal effects on sugar recovery (about 4% of sugar losses) (Kamal et al., 2011). In fact, a significant reduction of fermentable sugars, c.a. 20 to 30%, have been obtained when using high activated charcoal charges (7.5–10%) (Wang and Chen, 2011; Lee et al., 2011). However, in this work, important reductions of aliphatic acids content were also obtained (acetic acid, 37%; formic acid 27%), whereas HMF, furfural and phenolic compounds were completely removed from the hydrolysates. The higher adsorbent power of the activated charcoal for furan derivatives compared to formic and acetic acids was previously reported (Lee et al., 2011). Several studies using this treatment for the detoxification of different hemicellulosic hydrolysates have obtained efficient results in removal of degradation products: Kamal et al. (2011) demonstrated a reduction of furfural (58%) and total phenolics (78%) with 2.5% activated charcoal; Vallejos et al. (2016) with the application of 3% activated charcoal method obtained 97, 81 and 100% of reduction for phenolic compounds, furfural and HMF, respectively, while acetic acid was not affected. A removal of 46% of acetic acid and 81% of phenolic compounds occurred when 8% charge of activated charcoal was used (Mateo et al., 2013).

Unfortunately, all employed methods caused losses of hydrolysate volume. For example, CaO treatment led to a reduction of c.a. 19% of the hydrolysate volume, which agrees with the values obtained by Mateo et al. (2013). Granular activated charcoal resulted in a hydrolysate loss of 10%, whereas the treatment with powder activated charcoal substantially affected the volume of the hydrolysate causing a decrease of c.a. 30%. However, it is worth to highlight this treatment enable a complete removal of the colour of the hydrolysates, in contrast to the small decrease in the colour of hydrolysate treated with granulated charcoal.

3.3. Fermentation

3.3.1. Lactic acid production by E. coli JU15 in mineral medium containing glucose or xylose

Fermentation of *E. coli* JU15 was performed in mineral AM1 medium containing glucose or xylose (20 g/L) as carbon source in order to observe the performance of the strain in a medium without the synergistic effects between inhibitors, sugars or solids fraction. Previous studies pointed out a good fermentability of JU15 in AM1 media with xylose, where was displayed 95% of the theoretical lactate yield and a productivity of 0.79 $g_{D\text{-}LA}$/Lh (Utrilla et al., 2012). The results shown in Fig. 1 (A and B) and Table 3 indicate

that although LA yields were similar in both sugars medium (about 0.96- 0.97 $g_{D\text{-}LA}/g_{Sugars}$), the growth rates (0.23 *vs* 0.08 μ; data not shown) and the LA volumetric productivity (0.9 *vs* 0.45 $Q_{D\text{-}LA}$) were quite higher when glucose was used. These results are in agreement with those pointed out by some authors (Utrilla et al., 2016) that used *E. coli* JU15 in minimal medium with 40 g/L of glucose or xylose. Glucose was consumed faster (completely depleted in 21 h of fermentation) whereas xylose was almost entirely consumed (95.3%) in 41 h of fermentation. During fermentation no acetate production was observed, on the contrary, there was a slight decrease in their concentration, mainly in the experiment with glucose. The results showed also that most active cell growth occurred during 16 h and 24 h when glucose and xylose were used as carbon source, respectively, being the most LA produced during the stationary phase (data not shown).

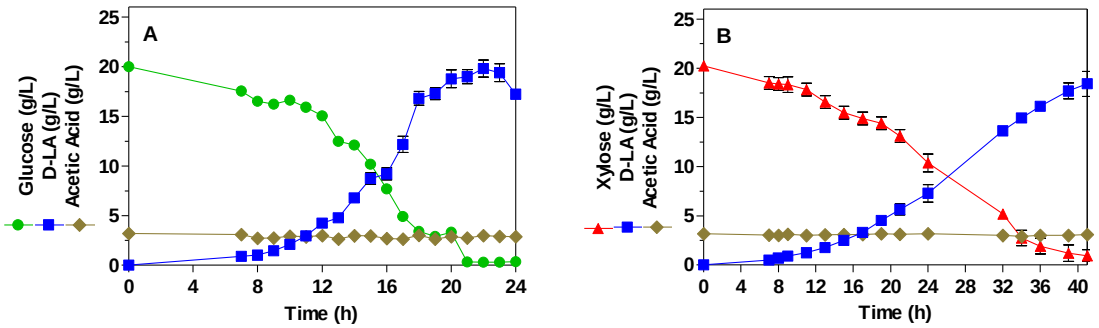

Fig. 1 Fermentation performance with recombinant strain *Escherichia coli* JU15 in mineral AM1 media (0.2 L fermentations) supplemented with glucose or xylose (20 g/L) as the carbon source and sodium acetate (4g/L): A) Fermentation kinetics for AM1 mineral medium plus glucose; B) Fermentation kinetics for AM1 mineral medium plus xylose

3.3.2. Effect of detoxification on D-lactic acid production

In order to examine the effect of the detoxification on fermentation of D-LA using the lactogenic strain JU15, the detoxified hydrolysates with 10% powder activated charcoal and the non-detoxified were tested. Both hydrolysates were adjusted at pH 7 with CaO (optimum level for fermentation) and supplemented with AM1 nutrients. Fig. 2 (A and B) shows the kinetics for sugars and inhibitors consumption, as well as D-LA production. Table 3 shows the fermentative parameters of bioconversion assays. In the detoxified hydrolysate, glucose and arabinose were almost completely consumed before 24 h. Nevertheless, in 12 hours, about 66% of glucose was consumed compared to only 4% of arabinose, despite the amount of arabinose that was about half of the glucose. Xylose consumption started after most of the glucose was consumed. This behaviour is in

agreement with previous reports where the strain JU15 was used to ferment glucose-xylose in mineral media or actual lignocellulosic hydrolysates (Utrilla et al., 2016). In fact, there was also an important xylose consumption in the first 24 hours (75%) and although glucose had been fully depleted before 48 h, xylose and arabinose remained at residual levels (≤ 0.3 g/L) until the end of the fermentation (60 h).

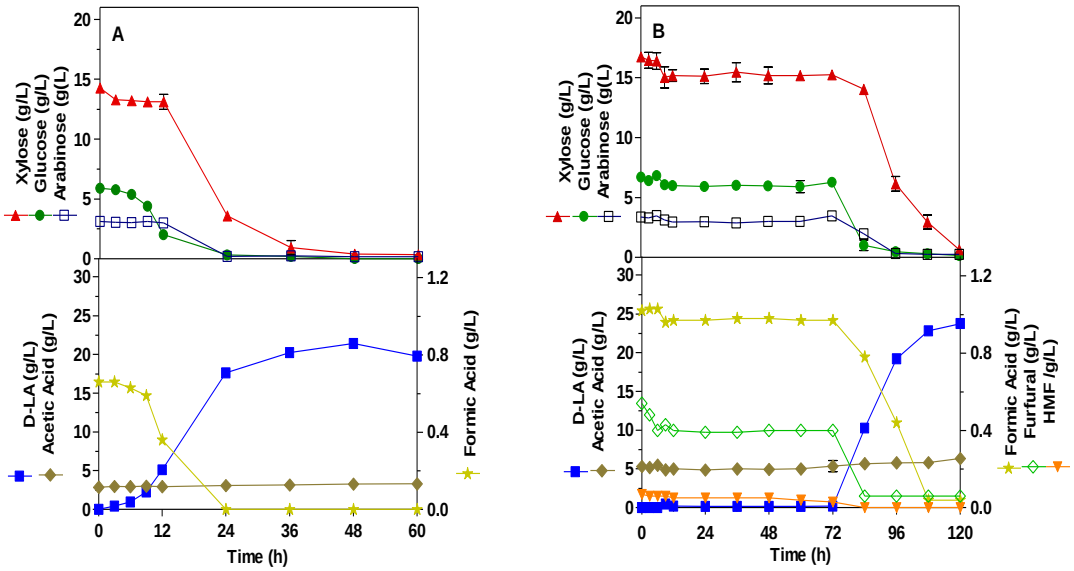

Fig. 2 Fermentation profiles for lactate production by strain *E. coli* JU15: A) Detoxified hydrolysate (with activated charcoal); B) Non-detoxified hydrolysate. These liquors were supplemented with mineral AM1 medium and fermentations were performed in mini-fermenters with 0.2 L and an initial inoculum of 0.076 g_{DCW}/L, at 37 °C, 100 rpm during 60-120 h. 75% of the total sugars are pentoses (xyl: ara, 5:1). Data points are the average of at least two independent replicates.

Regarding non-detoxified spent liquors, a behaviour very different was observed, where only after 72 h the lactogenic bacteria started effectively consuming sugars. This behaviour suggests an adaptation process to the whole culture – hydrolysate media composition, with a 72 h lag phase. As shown in Fig. 1 (C), the fermentation profile of the minimum medium demonstrated that *E. coli* JU15 requires a longer time for consumption of xylose. The consumption rate of xylose and arabinose increased with the glucose depletion. This behaviour is according with those presented by Utrilla et al. (2016) using strain JU15 in simulated hydrolysates, where after glucose depletion, a simultaneous consumption of xylose and arabinose was observed, achieving a total reduction of the sugars (62 g/L) after 72 h. These same authors used sugar cane bagasse hydrolysate containing AM1 media reduced (1 mM betaine, 0.1 g/L sodium citrate and 0.85 g/L of ammonium phosphate salts) and obtained a depletion of all sugars (51.14 g/L) at 72 h.

As can be seen in Table 3, the highest lactic acid concentration was obtained in the non-detoxified liquor (23.8, in comparison to 21.4 g/L in the detoxified liquor), since

these liquors presented a higher amount of sugars (about 27 *vs*. 23 g/L). Nevertheless, the lactic acid yield on consumed sugars attained 0.94 $g_{D\text{-}LA}/g_{Sugars}$ for detoxified hydrolysates and 0.92 $g_{D\text{-}LA}/g_{Sugars}$ for non- detoxified liquor (Table 3). Thus, when strain JU15 was used, the detoxification method of CLRext hydrolysate improved fermentation performance in terms of volumetric productivity but not on the initial quantity of sugars. Apparent yields above the theoretical maximum, due to non-quantified sugars, have been determinate in the sugar cane bagasse hydrolysates using *E. coli* JU15 (Utrilla, et al. 2016). Other authors have utilized different engineered strains of *E. coli* for lactic production: Wang et al (2013b) demonstrate a yield of 85% and maximum productivity of 1.18 g/Lh of L-lactic acid from sugarcane molasses and corn steep liquor by *E. coli* WYZ-L; Liu et al. (2014) obtained D-LA yield of 97% on a pilot scale using glucose mineral medium and Ca(OH)2 for neutralization by *E. coli* HBUT-D.

Table 3. Effect of media composition and fermentation mode on lactic acid production

	Sugars (g/L)	***Time[b] (h)***	***Saccharification yield (%)***	***Lactic acid (g/L)***	***Y_{LA} (g/g)***	***Q_{LA} (g/L.h)***
AM1 medium plus xylose	20.0	22	-	19.8	0.97	0.90
AM1 medium plus xylose	20.0	41	-	18.4	0.96	0.45
Non-detoxified hydrolysate[a]	27.0	120	-	23.8	0.92	0.20
Detoxified hydrolysate[a]	23.3	48	-	21.4	0.94	0.45
SHF	29.9	60	74.9	28.0	0.96	0.47
SSF	21.2	60	53.7	20.7	0.98	0.34
SHCF	37.9	60	37.2	34.0	0.99	0.57

[a] 75% of the total sugars are pentoses (xyl: ara, 5:1); [b] time to reach the highest lactic acid concentration Q_{LA} lactate productivity, Y_{LA} lactate yield on sugars consumed

It is important to highlight that while the maximum concentration of D-LA was reached at 48 h for detoxified hydrolysates, for the non-detoxified spent liquor, the highest lactic acid content was achieved at 120 h. As result, global D-LA volumetric productivity in the detoxified liquor is more than double when compared to non-detoxified hydrolysate (0.45 $g_{D\text{-}LA}$/Lh *vs* 0.20 $g_{D\text{-}LA}$/Lh). However, with the non-detoxified hydrolysate all D-LA was produced from 72 to 120 h (when sugars were consumed, and furans concentrations decreases); then if the volumetric productivity is calculated for such period a value of 0.56 $g_{D\text{-}LA}$/Lh. These results suggest that strain JU15 is a robust microorganism that can contend with an initial and combined concentration of 0.8 g/L of furans, 1.7 g/L of total phenolics, 5 g/L of acetic acid and 1.1 g/L of formic acid, and after an adaptation period

can efficiently ferment (in terms of yield and volumetric productivity) a combination of sugars into D-LA.

The inhibition of sugar consumption and lactic acid production, in the non-detoxified liquor in the first 72 h could probably be attributed to phenolic compounds, formic acid and to furan derivates. Furan derivatives affect different intracellular pathways, extending the lag phase and phenolic compounds can alter biological membranes harming growth rates as well as the action of hydrolytic enzymes (Moreno et al., 2013). The low productivity of D-LA in concentrated hydrolysate from sugar cane bagasse fermented by JU15 in a pilot scale fermenter was associated to a high concentration of acetate (above 12 g/L), the presence of furans, phenolic compounds and the low initial cell density (Utrilla et al., 2016). In fact, in this work, only from 84 h, LA was detected in non-detoxified hydrolysates, at the same time which it was observed an important reduction of furfural and formic acid. Also, a previous study has shown that bacterial strains, including *E. coli* are able to metabolize furfural and 5-HMF, reducing them to their corresponding alcohols (Boopathy et al., 1993). Phenolic compounds gradually decreased (ca. 50%) from start to finish of the fermentation (data not shown).

Regarding aliphatic acids, formic acid was significantly reduced (96%), whereas acetic acid had an increase of 21% during fermentation of the non-detoxified hydrolysates. For detoxified liquors, 0.8 g/L of formic acid was depleted in 24 h of fermentation and acetic acid had an increase of 15%. The production of acetic acid by *E. coli* JU15 as fermentation by-product has been reported by Utrilla et al. (2016) with an increase of approximately 40% in relation to initial acetic (12.4 g/L) in concentrated hydrolysates. Therefore, the tolerance or metabolization of all these toxic compounds by JU15 is an important advantage, since could simplify the fermentative process avoiding the detoxification step.

3.3.3. SHF, SSF and SSCF processes

In order to compare LA production in SHF and SSF, glucan-rich solids (5% of solid loading) alone or together with hydrolysates, three different modes of fermentation were carried out. These solids that contained about 74% glucan, 6% xylan and 16.8 lignin were hydrolysed with 20 FPU/g solid of a cocktail enzymatic (Novozymes). SHF process was performed by 72 h, while SSF and SSCF were preceded by a pre- saccharification step of 12 h for a period of action of the enzymes. The results of enzymatic saccharification are presented in Table 3. Glucan-rich solids released 75% of glucose in 72 h. These results were similar to enzymatic saccharification reported for the delignified

solids based on the Ghose protocol (64 FPU/g glucan of cellulase 1.5L and 60 IU/g pNPG units/g glucan of β-glucosidase) (Alves-Ferreira et al., submitted). Fernandes et al. (submitted) found values of enzymatic digestibility ranging from 40 to 75% for cellulose-rich solids of CLR, where solid loading (2–10%) and enzyme loading (6.34–23.66 FPU/g solids) had a marked effect in saccharification, since the higher saccharification yields occurred when solid loading was low and enzyme loading was the highest.

Regarding SSF and SSCF, a glucan recovery of 53.7% and 34.6% were obtained in 12 h of hydrolysis, evidencing a higher conversion of the glucan by SSF process (c.a. 44%) to the detriment of SSCF. An increase in the enzyme dosage could probably improve the hydrolysis of CLR glucan as already shown in our previous studies using another enzyme (Accellerase 1000 - Genencore Inc.) (Fernandes et al., submitted). Nevertheless, these results suggest also that the presence of the detoxified hydrolysed liquor together with the solids during pre-saccharification might be a limiting factor for glucan conversion, since the presence of sugars may have inhibited the enzymatic activity. Monomeric sugars, acetic acid, furans, and especially phenolic compounds were shown to have inhibitory effects on cellulase activity during cellulose hydrolysis (Kim et al., 2011; Xiao et al., 2004). In fact, mass transfer limitations away from the enzyme have been also reported, but with inhibition more apparent at high-solids loadings (Modenbach and Nokes, 2013; Hodge et al., 2008). Meantime, xylan hydrolysis reached values of 72, 41. and 33% for SHF, SSF and SSCF, respectively (data not shown).

Hydrolysates based media were inoculated with lactogenic strain *E. coli* JU15 (c.a. 0.076 g_{DCW}/L) and supplemented with AM1 nutrients, 1.0 mL/L of betaine HCl (1 M) and 1.0 mL/L Kanamycin (30 mg/mL), being SHF and SSF also supplemented with 4 g/L sodium acetate. During fermentation, the solids were not removed. Fermentative processes in the presence of solids using ethanologenic *E. coli* have been described with success (Vargas-Tah et al., 2015). For SSCF besides solids was also included the hemicellulosic hydrolysate. Efficient utilization of these two sugars is a prerequisite for cost-effective conversion of lignocellulose to added-value chemicals (Ye et al., 2014). Time courses of sugar consumption, as well as the production of aliphatic acids and D-lactic acid in the slurries from CLR, are shown in Fig. 3 (A-C). Initial sugar concentration in the slurry, after saccharification step and the addition of nutrients solutions, was of 29.85 g/L, 21.15 g/L and 37.93 g/L, respectively for SHF, SSF and SSCF (Table 3). Glucose was used up within 36 h in all the processes, including SHF that presented approximately 40% more glucose compared to other two modes. The consumption rate of glucose was of 0.77 g/Lh (SHF), 0.56 g/Lh (SSF) and 0.54 g/Lh (SSCF) (data not shown). However, the most productive period started at 12 h of fermentation.

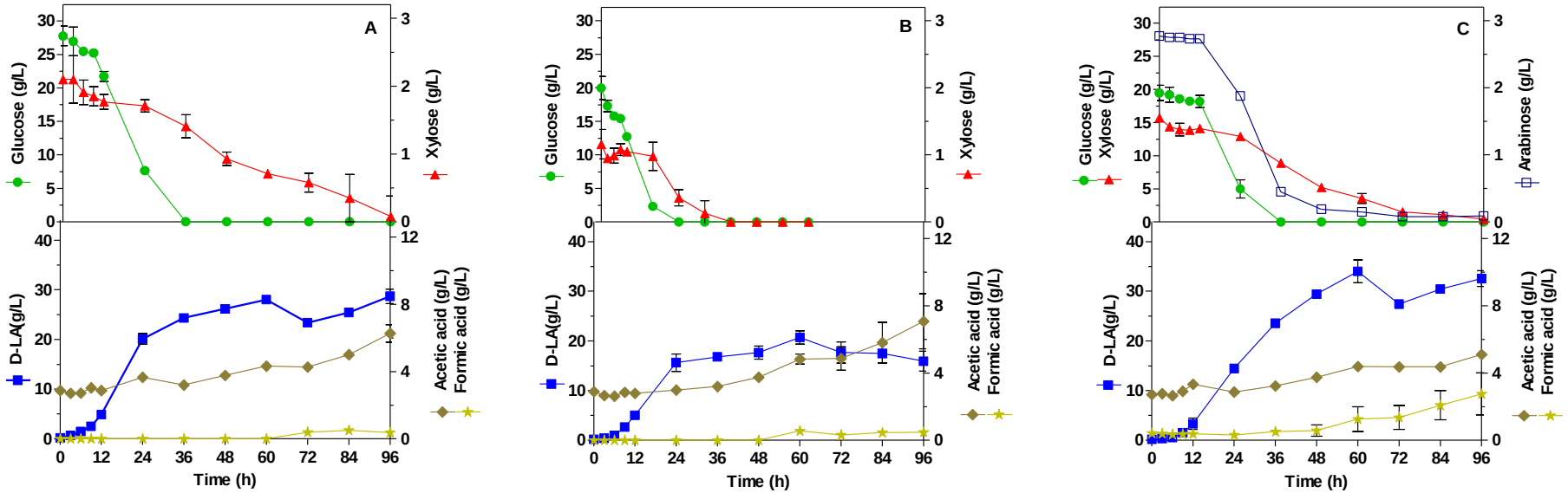

Fig. 3 Fermentation profiles by lactogenic strain *E. coli* JU15, using CLR subsequently treated by autohydrolysis and 4% NaOH, at 130 °C for 1 h: A) Separate Hydrolysis and Fermentation (SHF); B) Simultaneous Saccharification and Fermentation (SSF); C) Simultaneous Saccharification and Co-Fermentation (SSCF) tested in combination with the detoxified hemicellulosic hydrolysates. The saccharification (72h) and pre- saccharification (12h) were performed in mini-fermenters using a solid loading of 5% at 50°C and 200 rpm. Fermentations were carried out in the same vessel and in the presence of solids with 0.2 L and an initial inoculum of 0.076 g_{DCW}/L, at 37 °C, 200 rpm during 96h. Data points are the average of at least two independent replicates.

Similarly, to what occurred in the fermentations using pentose rich hydrolysates, the consumption of xylose has become more efficient when there was already a low amount of glucose in the medium. Other authors have been reported a consumption almost simultaneous of glucose and xylose by lactogenic strains (Ye et al., 2014). It is important to note that, even in small amounts, were required 60 h for the consumption of xylose in SSF and 96 h in SHF. In the SSCF process 97% of xylose was consumed within 96 h, but the amount of xylose consumed corresponded to 15.3 g/L. In fact, the consumption of xylose from detoxified hemicellulosic hydrolysates was faster compared to consumption of residual xylose from glucan solids. The presence of solids is challenging due to mass transfer limitations, low water activities or insufficient mixing (Kim et al., 2011). The arabinose present in SSCF showed a similar behaviour to xylose when only started its metabolization after a significative decrease of glucose.

Considering the D-LA yields, the SSF exhibited a good performance (0.98 $g_{D\text{-}LA}/g_{Sugars}$). Nevertheless, the maximum concentration of D-LA was only of 20.7 g/L, with a volumetric productivity of 0.34 $g_{D\text{-}LA}$/Lh as a consequence of the lower available fermentable sugar content. During fermentation, does not appear to have occurred an important release of sugars. This fact results mainly in the fermentation conditions (temperature and pH), which are not operated to the optimal level of the enzymatic activity. For the same reasons, for SSCF the enzymatic activity after the beginning of the fermentation was not also effective and most of the sugars present in the fermentative medium come from the hemicellulosic hydrolysates. Thus, a maximal D-LA concentration of 34 g/L was achieved within 60 h, resulting in a productivity of 0.57 $g_{D\text{-}LA}$/Lh and a yield of 0.99 $g_{D\text{-}LA}/g_{Sugars}$. Different microorganisms have been applied in the LA production from lignocellulosic feedstock using SSF and SSCF strategies: Hama et al. (2015) showed that a combined process employing short-term milling and SSF from hardwood pulp and using the *L. plantarum* mutant resulted in a D-lactic acid yield of 0.88 g/g_{sugars} with a lactic acid titre of 102.3 g/L; another study conducted by Hu et al., (2014) presented a yield of 0.68 g/g of LA from NaOH pretreated non-sterile corn stover in the fed-batch SSF process by *B. coagulans* LA204; yields of 79.7%, 69.6%, and 64.7% from 15%, 20% and 25% (w/w) solids content dry dilute acid pretreated corn stover, respectively and using *P. acidilactici* ZY15 in SSCF process have been also reported (Qiu et al., 2017).

Regarding SHF, 28.05 g/L of D-LA was reached, giving a lactic yield on consumed sugars of 0.96 $g_{D\text{-}LA}/g_{Sugars}$ and a volumetric productivity of 0.47 $g_{D\text{-}LA}$/Lh within 60 h. These results show that SHF produced a higher amount of LA compared to SSF, due to a better hydrolytic performance, which occurred separately from fermentation. In general, SSF present advantages over SHF, since glucose released during enzymatic hydrolysis is simultaneously converted to the end product (Iyer & Lee, 1999), without

inhibiting enzyme action. Indeed, a high sugar concentration in the medium negatively affects fermentation rate and bacterial growth due to an imbalance in osmotic pressure (John et al., 2009). Thus, SSF can improve the process fermentative in relation to residence time and lactic acid concentration in the medium (Hama et al., 2015). Nevertheless, SHF has been also described as a strategy more efficient than SSF for L-lactate fermentation, since cellulase is inhibited by lactic acid concentration (Wang et al., 2013a). In this work, SSF process presented a fermentative performance slightly higher than SHF, but in fact, the poor hydrolytic performance due to the operating conditions already mentioned, did not allow a greater success in the use of this approach. Anyway, strain JU15 is capable of producing LA with efficiency even in the presence of solids (and as described above in this section, even in the presence of acetic acid, formic acid, furfural, HMF and a relatively high amount of phenolic compounds) and considering the hexoses and pentoses available in the medium. However, if we take into account the lactic acid production potential from the glucan content in the solids, it is concluded that the overall process using SSF and SSCF are not very efficient. Indeed, one of the factors that allow the success of SSF is related to the optimal conditions of operation for enzyme and bacteria and this was not possible using *E. coli* JU15.

The initial acetate concentration was similar in all the slurries (about 3 g/L) and during fermentation, there was an increase from 1.4 g /L to 2 g/L of this compound. The decrease in the production of L-lactic acid with increasing concentration of sodium acetate using a genetic engineering of *E. aerogenes* also have been observed (Thapa et al., 2017). However, according to Fernández-Sandoval et al. (2012), the growth of ethanologenic derivatives of strain JU15 was improved in the presence of 5 g/l sodium acetate in the culture media. Regarding formic acid, it was produced during fermentation, contrary to what occurred in hemicellulosic hydrolysates. In SHF and SSF it was possible to observe the presence of this compound from 60 h and 72 h, respectively; in SSCF, formic acid decrease until 24 h and then start to increase reaching 1.36 g/L within 72 h. These results show that the JU15 strain can show a different behaviour in the presence of solids at the expense of hydrolysates, exhibiting, in the case of xylose, a slower metabolism or in the case of formic acid, its production instead metabolization

Outlook

Overall, the results indicate that selected diluted acid hydrolysis conditions (121 ºC, 15 min, 3% H_2SO_4) can efficiently convert oligosaccharides present in hemicellulosic spent liquor from CLRext into monosaccharides (mainly xylose). The powder activated

charcoal is the more efficient treatment for these hydrolysates, since remove all furan derivatives, phenolic compounds and an important part of the aliphatic acids. Considering the advantages presented by each fermentation mode and targeting the process economics, the non-detoxified hydrolysates fermentation and SHF process suggested being the most advantageous option, even though the fermentation time of the non-detoxified liquor is higher, although this might be overcome by higher initial cell concentration. In addition, activated charcoal detoxification (followed by pH adjustment) cause a significant reduction in the volume of the liquor, that together with the decreased in sugar concentration implies a significant loss of sugar availability. As far as SHF is concerned, the hydrolysis of glucan-rich solids released 75% of free glucose in 72 h, while the pre-saccharification led to values from 37 to 54% of glucan hydrolysis. The inclusion of the hemicellulosic liquid fraction resulting of the autohydrolysis together with NaOH pretreated solid in the same fermentation vessel increases the amount of fermentable sugars but decrease the release of sugar during enzymatic hydrolysis.

4. Conclusions

Sequential processing, autohydrolysis, delignification, sulfuric and enzymatic hydrolysis and fermentation of CLR it proved to be an efficient strategy for the selective fractionation of this unexplored residual biomass providing hemicellulosic and cellulose–rich stream, free sugars and lactic acid in a framework of biorefineries. Both pentoses (from hemicellulose) and glucose (from cellulose) can be efficiently used as substrate to produce D-lactic acid by modified *E. coli* JU15, with yields from 92 to 99%. Furthermore, *E. coli* JU15 is able to metabolize glucose and xylose in the presence of moderate amounts of furans, formic acid, acetic acid and phenolic compounds present in non-detoxified hydrolysates maintaining high lactate yields.

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CAPÍTULO VIII

Conclusões e Considerações Finais

Conclusões e Considerações Finais

Neste trabalho desenvolveu-se uma estratégia experimental para a valorização integrada dos resíduos da esteva (*Cistus ladanifer*), provenientes de destilarias de óleos essenciais, a fim de evidenciar o potencial e a viabilidade desse subproduto como matéria-prima para as biorrefinarias. O estudo de um conjunto de processos sequenciais (extracção, pré-tratamento hidrotérmico, deslenhificação, hidrólise química e enzimática e fermentação) permitiu definir a via de exploração, na qual é possível obter seletivamente açúcares hemicelulósicos e celulósicos para a produção de ácido láctico e um conjunto relevante de compostos bioativos a partir da lenhina e dos extrativos.

Seguidamente verse-á em detalhe os principais resultados obtidos.

As variações na composição química das diferentes partes morfolóficas de C. ladanifer e potencial do seu teor de extrativos

A composição química dos diferentes componentes da planta (caules, ramos, folhas e cistos) apresentou variações importantes, em especial, nos teores de extrativos e hidratos de carbono. Em síntese, os componentes com maior concentração de extrativos exibem um menor teor de polissacarídeos e lenhina. Assim, podem ser encontradas quantidades significativas de extrativos nos cistos, nos ramos e, sobretudo, nas folhas, enquanto que o caule é a parte morfológica que exibe a maior concentração de polissacarídeos, seguido de lenhina. A partir dessa análise, é possível afirmar que a alta concentração de extrativos presentes também nos resíduos de destilaria (40 g/g de material seco) ocorre, principalmente, devido à contribuição das folhas. De facto, é neste componente que estão concentradas as maiores quantidades de óleos essenciais, logo, é dada prioridade à parte áerea da planta nos processos de destilação.

Em face disso, conclui-se que uma das principais vias de exploração dos resíduos da esteva, assenta na valorização dos extrativos como fonte de fitoquímicos. A avaliação desses compostos indicou uma presença relevante de compostos fenólicos, tais como flavonóides e taninos, e também uma alta atividade antioxidante. A extração sucessiva por Soxhlet, usando solventes como diclorometano, etanol e água, é altamente eficaz para a extração daqueles compostos, sendo os extractos em etanol os mais interessantes compostos fitoquímicos dessa espécie. Desse modo, após a remoção dos extrativos, obtém-se uma biomassa lenhocelulósica remanescente com teores mais interessantes de polissacarídeos e lenhinas com potencial uso para outras aplicações.

Auto-hidrólise usando biomassa extratada vs. biomassa não extratada apresenta vantagens relevantes

O processo de auto-hidrólise, sob condições não-isotérmicas, demonstrou ser eficiente para a hidrólise das hemiceluloses e produção de oligossacarídeos usando duas abordagens: i) biomassa residual obtida directamente das destilarias (i.e com extrativos) e ii) biomassa livre de extrativos. Entretanto, alguns pontos importantes devem ser destacados, visto que essas diferentes abordagens se traduzem em licores com concentrações de compostos bastante diversas entre si. Considerando-se a condição optimizada, correspondente ao rendimento em xilo-oligossacarídeos (XOS) mais elevado e sólido rico em glucanas (log R_0 3.51¸ 205 °C), constatou-se uma maior concentração de monossacarídeos e compostos inibidores nos licores provenientes da biomassa não extratada (respetivamente em g/L, 6,6 *vs* 17 e 12,2 *vs* 5,6). Nesta condição, a maior parte dos extrativos que são solubilizados em água, usando o processo Soxhlet, foram assim removidos pelo tratamento hidrotérmico. Daqui conclui-se que alguns dos açúcares determinados na fração líquida da primeira abordagem são provenientes dos extrativos e não das hemiceluloses e/ou celulose, uma vez que tais concentrações ficaram acima do esperado, tendo em vista a composição inicial da matéria prima. Além disso, os valores relevantes de glucose, inclusive nas condições mais moderadas de temperatura, não é expectável em tratamentos de auto-hidrolise, cujo objetivo maior é a hidrólise das hemiceluloses e não da celulose. Não obstante, a remoção dos extrativos antes do pré-tratamento hidrotérmico, usando etanol e água, resultou num rendimento em XOS mais elevado e numa menor concentração de compostos inibidores, em especial de compostos fenólicos. Este aspecto é muito importante, visto que concentrações elevadas desses compostos podem aumentar os possíveis efeitos de toxicidade durante a fermentação. Acresce ainda que a recuperação de uma quantidade tão relevante de extrativos abre mais uma via de exploração com grande interesse, tendo em conta a ampla gama de compostos de valor acrescentado presentes nesses extratos. Posto isso, removê-los antes do pré-tratamento hidrotérmico é o procedimento mais apropriado para uma produção de extratos e de licores hemicelulósicos com maior pureza, bem como de sólidos com rendimentos em celulose e lenhina mais elevados. Cabe frisar, ainda, que a alta densidade dos resíduos de esteva, comparativamente a outros materiais como, por exemplo, as palhas, permitiu empregar uma razão líquido/sólido baixa (6:1) durante o processo hidrotérmico, resultando, assim, num hidrolisado mais concentrado. Esta particularidade também contribui para menores gastos de água e energia, o que, torna-se muito vantojoso do ponto de vista ambiental e económico.

O rendimento de deslenhificação, a qualidade da lenhina e a recuperação de compostos fenólicos estão relacionados ao tipo de processo e a natureza do agente deslenhificante

Relativamente aos tratamentos de deslenhificação, os resultados obtidos sugeriram que a natureza do agente deslenhificante e/ou a concentração do agente alcalinoi afetam o grau de remoção e a qualidade da lenhina. O tratamento alcalino, utilizando soluções de hidróxido de sódio em várias concentrações apresentou a maior eficiência na remoção da lenhina, seguido pelo processo organosolv usando misturas glicerol/água em meio alcalinizado com hidróxido de sódio. Em ambos os tratamentos, o grau de deslenhificação aumentou com o aumento da concentração do hidróxido de sódio. Por outro lado, no que se refere ao tratamento apenas com hidróxido de sódio, as remoções da lenhina não foram afetadas pelo tempo de residência. Além disso, convém ressaltar que as percentagens de deslenhificação e hidrólise enzimática mais baixas apresentadas para a biomassa não tratada comparativamente aos sólidos pré-tratados (considerando as mesmas condições), mostram que a remoção das hemiceluloses do sólido, antes da aplicação de processos alcalinos, melhora o rendimento da deslenhificação e subsequente conversão da celulose, sendo esta mais uma vantagem atribuída ao processo hidrotérmico. A tecnologia organosolv usando etanol e água como solventes, apresentou pouca eficiência na deslenhificação dos resíduos de destilaria da esteva, seja com adição de catalisadores, como sais inorgânicos ou até ácido sulfúrico, mesmo com o aumento do o tempo de reação. Em consequência , os sólidos remanescentes desse processo também não obtiveram resultados satisfatórios de hidrólise enzimática ao contrário dos obtidos a partir dos meios alcalinizados. Os compostos fenólicos derivados da lenhina extraídos pelo tratamento com etanol e água também foram diferentes daqueles encontrados nos meios alcalinizados.

As alterações estruturais na fração sólida remanescente e nas lenhinas removidas, decorrentes dos processos de fracionamentos, foram avaliadas por meio da determinação das diferentes unidades de siringilo, guaiacilo e hidroxifenilo (S:G:H) da lenhina, bem como dos produtos de pirólise derivados de polissacarídeos. A auto-hidrólise promoveu nos sólidos pré-tratados uma redução dos polissacarídeos derivados de pentoses e um enriquecimento das unidades S e H. Não obstante, a composição residual da lenhina nos sólidos deslenhificados (ricos em celulose) indicou uma maior susceptibilidade das unidades S às reações de deslenhificação, dado que estas foram preferencialmente removidas em comparação com as unidades G e H, resultando numa diminuição na relação S/G em todas as amostras analisadas. Evidencia-se, ainda, uma maior semelhança entre os sólidos resultantes da auto-hidrólise e do processo

organosolv utilizando etanol em relação às características composicionais dos derivados de polissacarídeos. Em ambas as amostras, os níveis de levoglucosana, principal composto formado a partir da pirólise de polissacarídeos, permaneceram altos, em contraste com os baixos níveis detectados nas frações de biomassa obtidas nos tratamentos conduzidos em meios alcalinos. Este facto pode ter ocorrido em virtude da natureza menos destrutiva dos polissacarídeos do etanol em relação ao hidróxido de sódio. As lenhinas precipitadas a partir dos licores organosolv apresentaram um maior rácio S/G comparadas às lenhinas resultantes dos tratamentos com hidróxido de sódio. Nestas últimas, o tempo de reação influenciou a composição monomérica das lenhinas, com um maior rácio S/G na amostra submetida a um maior tempo de reação. A lenhina obtida com glicerol em meio alcalino apresentou um maior teor de hidratos de carbono residual, talvez pela maior dificuldade apresentada no processo de lavagem dessa amostra, em função da viscosidade do solvente.

É pertinente afirmar que os diferentes tipos de tratamento interferem também no aspecto visual das lenhinas precipitadas: a tecnologia organosolv utilizando etanol/água produziu lenhinas mais macias e menos escurecidas; as lenhinas do processo organosolv com glicerol apresentam um aspecto viscoso, necessitando de bastante lavagem para libertar o glicerol preso ao material; já as lenhinas produzidas no processo usando soda são bastante escuras e relativamente duras quando secas, assemelhando-se a pequenos pedaços de carvão.

As pentoses e glucose podem ser usadas eficientemente como substratos para a produção de ácido láctico pela estirpe transformada Escherichia coli JU15

Os licores hemicelulósicos obtidos no tratamento hidrotérmico foram submetidos a ensaios de pós-hidrólise com ácido diluído para a obtenção de um rendimento elevado de monossacarídeos preconizando uma menor formação de inibidores. A condição otimizada permitiu a obtenção de licores apropriados para serem utilizados em processos fermentativos, valendo-se de uma menor concentração de ácido sulfúrico e menor tempo de reação, quando comparado ao ensaio padrão.

Os hidrolisados foram sujeitos a processos de destoxificação com carvão ativado para comparação de desempenho fermentativo em relação aos hidrolisados não destoxificados (ajustado a pH 7). A utilização de diferentes tipos de carvão ativado (granulado e em pó) demonstrou ser muito eficiente na remoção dos compostos inibidores, como furfural, hidroximetilfurfural e, em especial, dos compostos fenólicos.

Entretanto, o carvão ativado em pó, o mais eficaz, conduziu a uma perda importante de açúcares e a um decréscimo significativo do volume total do licor.

Os sólidos ricos em celulose utilizados nos processos de fermentação foram deslenhificados usando o processo alcalino (NaOH 4%, 1 h), visto que nesta condição podem ser obtidos sólidos com taxas de deslenhificação e de hidrólise enzimática similares àquelas apresentadas pela condição operada com maior tempo de reação (NaOH 4%, 2h).

O desenvolvimento do processo de produção do ácido D-láctico consistiu num conjunto de ensaios envolvendo a utilização dos licores de pentoses com e sem destoxificação, e diferentes modos de fermentação, i.e., hidrólise e fermentação sequencial (SHF), sacarificação e fermentação simultâneas (SSF) sacarificação e co-fermentação simultâneas (SSCF) dos sólidos ricos em celulose em combinação com os hidrolisados hemicelulósicos destoxificados. Para a fermentação foi utilizada uma estirpe de *Escherichia coli* geneticamente modificada, nomeada como JU15 e com capacidade de utilizar pentoses e produzir apenas ácido D-láctico. Os ensaios biológicos envolvendo essa estirpe foram conduzidos em micro-biorreatores, com controle de pH por meio da adição automática de base. Desse modo, é garantida a manutenção das condições ótimas de cultura como pH, temperatura e agitação.

Todas as abordagens fermentativas apresentaram altos rendimentos de D-lactato (0,92–0,99 $g_{D\text{-}LA}/g_{açúcar}$), com uma conversão quase quantitativa dos açúcares a ácido láctico.

Em termos de economia do processo e aproveitamento do potencial de açúcares, cabe pois concluir que a fermentação dos hidrolisados não destoxificados e o processo de hidrólise e fermentação separadas afiguram-se como as opções mais favoráveis.

Em relação à fermentação utilizando licores não destoxificados, fica patente que o tempo de fermentação é significativamente prolongado quando comparado aquele em que se utilizam licores destoxificados. Mas, em contrapartida, estes hidrolisados apresentam uma maior concentração de açúcares fermentescíveis, e sofreram uma perda menor no seu volume final com o ajuste de pH, além de não exigirem gastos com o carvão ativado, usado no processo de destoxificação. É de se referir, ainda, que o uso de carbonato de cálcio para ajuste do pH permitiu também remover uma parte dos compostos fenólicos presentes nesses hidrolisados, o que se presume ter sido uma vantagem para o processo de bioconversão.

Quanto à estratégia SHF, a principal vantagem dessa abordagem assenta na obtenção de melhores resultados de hidrólise enzimática em relação à SSF, resultando, no final, em maiores concentrações de ácido D-láctico. De facto, em SSF, a hidrólise das

glucanas limitou-se, praticamente, à fase de pré-sacarificação, sendo este desfecho previsível ao considerar-se a ausência das condições ótimas de temperatura para a eficiência da atividade enzimática.

A conjugação da fração líquida hemicelulósica com os sólidos ricos em celulose para o estabelecimento da estratégia SSCF aumentou, a quantidade total de açúcares fermentescíveis no mesmo reator. Contudo, é necessário sublinhar que na presença dos hidrolisados a taxa de hidrólise das glucanas não foi significativa, exibindo rendimentos ainda mais baixos que os apresentados durante a pré-sacarificaçao de SSF que, por sua vez, ocorreu sem a presença do hidrolisado. Neste caso, possivelmente, houve uma inibição das enzimas pelos açúcares monoméricos já presentes no licor.

Os resultados também evidenciaram que a presença do material sólido no meio fermentativo não comprometeu a obtenção de bons rendimentos de D-lactato pela estirpe JU15. Além disso, é importante ressaltar que essa estirpe foi capaz de metabolizar os derivados de furano e parte dos compostos fenólicos presentes nos hidrolisados não destoxificados, mantendo os bons rendimentos de lactato.

Sugestões para trabalhos futuros

Tendo em conta estas considerações e face à perspectiva de utilização dos resíduos de destilaria da esteva, à escala comercial, sugere-se a complementação deste estudo com as seguintes linhas de investigação:

- Avaliação aprofundada da composição dos extratos da esteva, uma vez que constituem uma parte significativa desses resíduos. Os extrativos apresentam composição variada e são necessárias pesquisas adicionais para determinar quais compostos são responsáveis pelas atividades farmacológicas, assim como os mecanismos de ação envolvidos e possíveis interações;
- Desenvolvimento e otimização de metodologias à escala piloto para a remoção dos extrativos, de maneira a aumentar a eficiência e a produtividade deste processo;
- Utilização da tecnologia organosolv aplicando outros tipos de solventes orgânicos (por exemplo: acetona, metanol, ácido fórmico, ácido acético, etc), visando a obtenção de resultados de deslenhificação mais promissores, tendo em conta o maior valor potencial das lenhinas obtidas por estes processos;

- Realização de ensaios de produção de ácido D-láctico com os hidrolisados não-destoxificados utilizando uma maior concentração de inóculo, com o intuito de diminuir o tempo de fermentação, e em hidrolisados não-destoxificados concentrados a fim de analisar a performance bacteriana em meios com teores mais elevados de furanos e fenólicos;
- Estudo do aumento de escala dos processos de fracionamento estudados e também dos ensaios de bioconversão à luz de critérios referentes à viabilidade económica e às questões ambientais;
- Estudos sobre a exploração sustentável de *C. ladanifer*, considerando os seus possíveis impactos sobre a fauna, flora, solo e paisagem, bem como a sua capacidade de fornecer matéria-prima para a operação de uma de biorrefinaria de pequena escala.